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# THE EFFECT OF FASTING ON THE SERUM PROTEIN CONCENTRATION OF THE RAT\*

WITH SPECIAL REFERENCE TO THE QUESTION OF THE EXISTENCE  
OF AN IMMEDIATELY UTILIZABLE CIRCULATING  
PROTEIN FRACTION.

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(Received for publication, February 5, 1935)

Though apparently made untenable by more modern theories concerning the formation and intermediary metabolism of proteins by way of the amino acids, their so called building stones, the hypothesis of a "circulating protein" which could be absorbed, carried to the tissues and there used directly, is one which has proved attractive to a number of investigators ever since it was first enunciated by Voit (1). This pioneer investigator drew his conclusions from the results of experiments published in 1866 (2) in which he showed that the amount of nitrogen excreted by a starving dog during the first few days of fasting was directly dependent on the level of previous protein ingestion. Voit cited experiments by Landois (3), who found that when blood was injected, the serum proteins were readily burned and the nitrogen excreted in the urine, while the presumably "organized" protein of the red blood cells was much more slowly destroyed. Forster (4) found that if serum alone was injected, its protein was rapidly destroyed.

All of this work was done before the masterly researches of Kossel, Hofmeister and Emil Fischer had elucidated the essential nature of proteins as complexes of amino acids, in the light of which the interpretation of Voit is by no means necessary to explain his results. Meanwhile further study has shown the allegedly "unorganized" protein of blood plasma to be a complex of separate substances of a fairly regular, though somewhat variable pattern and with definite functions of its own entirely unconnected with protein transport. One of the most important of these functions was enunciated by Starling (5), who in the latter part of the 19th century published evidence that tissue fluid regulation was due to a balance between the colloidal osmotic pressure of the plasma proteins and the hydrostatic pressure of the blood flow in the capillaries. Since Starling's paper, the attention of investigators of the blood proteins has been centered largely on various aspects of this problem, with valuable additions to existing knowledge of edema in nephritis, malnutrition and various other diseases. Too many excellent reviews of this

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aspect of the problem (6) are available to make it necessary to discuss it further here.

In a recent paper Whipple and his coworkers (7) found that dogs receiving only sugar by mouth could be maintained practically in nitrogen equilibrium by means of intravenous injection of normal dog plasma. Plasma protein fed by mouth showed the same general reaction but the urinary nitrogen excretion was slightly higher. These writers interpret this as meaning that the injected protein is utilized a little more completely for forming new body protein, and suggest that the difference may be due to deaminization of the protein given by mouth. They feel that the injected protein must be utilized by the body, and that if this can happen in this emergency it may be suspected that normally there is a certain amount of give and take between body protein and plasma protein.

One need not question the authors' general conclusion that the body can use material coming from one body protein to fabricate badly needed protein material of different character, to point out, first, that the work presented throws no light on the method by which the transfer took place, and second, the fact that the utilization of a surplus quantity of *foreign* protein is not evidence that the organism's own plasma protein, present in only normal amounts, will be similarly used.

Bloomfield (8) recently reported studies on the effect of long continued low protein diet on the plasma protein level. Working with the white rat, he found that on a diet adequate in calories, minerals and vitamins, but containing only minimal amounts of protein, there was a small but definite drop in the serum protein level, occurring within the first week, with no further drop even when the low protein diet was continued for 21 weeks. Similar results were obtained when the animals were fasted for 3 weeks except for water.

In summary, it appears clear that animals receiving a diet inadequate in protein show a small initial drop in the level of the blood proteins. It also is evident that proteins injected into the blood stream are available in the metabolism of the recipient, and that such injected protein is perhaps more efficient even than the same protein fed by mouth. No data are available, however, to explain the mechanism of either the maintenance of the blood proteins following the small initial drop or the utilization of ingested protein.

Bloomfield's work was done on pooled sera, and therefore the factor of individual variation was not studied. Also, his data for the first few days of the experiments, which is apparently the critical time, were not as complete as appeared desirable. It was, therefore, determined to extend the tests in order to analyze more accurately what happens to the plasma proteins during the period immediately following the elimination of protein from the diet. It was felt that

such an experiment would provide conditions more closely approaching normal without such complicating factors as plethora, extraordinarily high plasma protein concentrations and the possible abnormal effects of a strange protein in the circulation.

#### EXPERIMENTAL

Healthy female rats of as nearly uniform size as possible were selected and divided into groups of five or ten. All had been on the laboratory stock diet (9) for their entire lives and were in an excellent state of nutrition. The animals were exsanguinated from the abdominal vessels as detailed by Bloomfield (8). The following observations were made: weight of animal; blood volume (as determined by exsanguination); total serum protein concentration. It was at first hoped to determine not only total proteins but to partition the albumin and globulin in individual rats. However, this proved impractical because of the small size of the blood samples obtained, particularly in the second series of experiments with young animals. Therefore separate groups of rats were killed and their sera pooled for the determination of the albumin-globulin ratios.

Two series of experiments were made. In both series the animals received nothing by mouth except water, which was freely available. In the first, large mature rats which had been discarded from the breeding stock of the laboratory were used. These animals averaged well over 200 gm. in weight at the beginning of the experiment. In this series a group of five animals on stock diet served as controls. Other groups of five or ten animals were killed after fasts of 1, 2, 3, 4, 5, 7, 9, 12 and 15 days. In the second series, young virgin females approximately 80 days old and weighing from 110 to 140 gm. were used. Groups of five were killed after fasts of 2, 3, 5, 7 and 9 days. The experiment had to be terminated at this time because of the death of the remaining animals. Whereas the old rats remained in excellent condition except for emaciation after a 15 day fast, the young animals looked decidedly ill after fasting 5 days and began to die on the 7th day.

Fasting, rather than low protein diet, was chosen as the condition of the experiment in order to make the protein deficiency as acute as possible and to obviate the protein-sparing action of carbohydrate.

Serum proteins were determined gravimetrically by the method of Barnett, Jones and Cohn (10). Separate determinations were made on the serum of each rat. Serum was chosen rather than plasma to avoid the variable withdrawal of water from the erythrocytes by oxalate or citrate, in accordance with the suggestion of Bloomfield. In determining the albumin-globulin ratios, globulin was precipitated by half saturation with ammonium sulfate in the usual manner, followed by gravimetric determination of the albumin in the filtrate by the same method as used for total proteins.

## RESULTS

Details of the experimental results are presented in the accompanying charts and tables. Figs. 1 and 2 show the individual total serum protein concentrations. Fig. 3 portrays the average total protein concentration in both series. Table I presents a summary of the data obtained in the series of young rats.

Study of these data confirms Bloomfield's finding of a small initial drop in the concentration of serum protein. This drop in the old

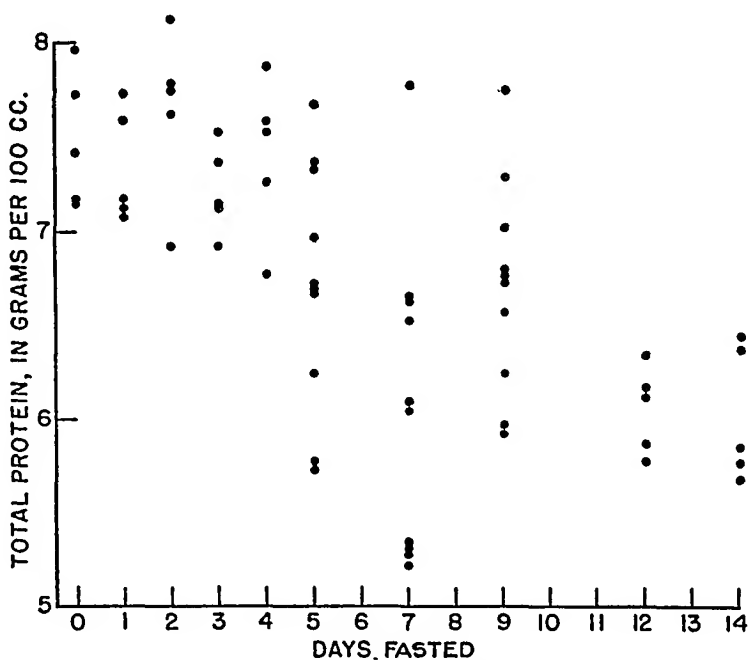


FIG. 1. Serum protein concentration in individual large mature female rats receiving only water. Each dot represents one animal.

rats is not obvious until the 5th day of fasting, but then it is very definite (see Fig. 3). It is also clear that no subsequent drop occurs up to 15 days of fasting. If the protein concentrations for the first 4 days, and then those of the succeeding periods, are averaged in order to smooth out minor variations, a drop of from an average of 7.65 to 6.60 is found. That is, there is a fall of about 14 per cent in the total protein. Similar results are obtained with the series of young animals except that here the initial level of the proteins was considerably lower, the drop is of less magnitude—from an average of

6.39 to 6.01, or about 7 per cent—and the fall is perceptible on the 3rd day.

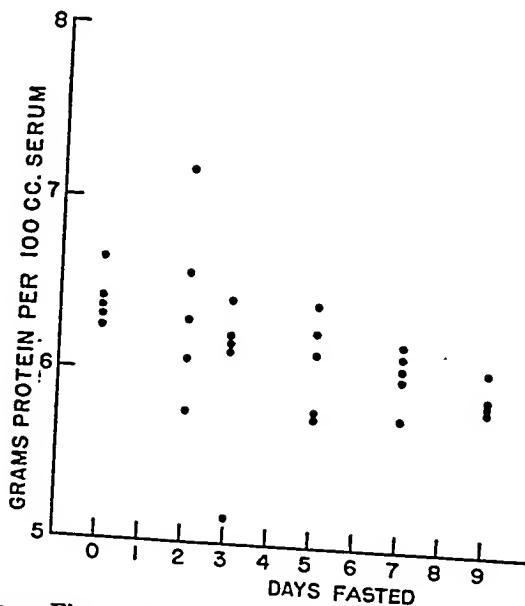


FIG. 2. Same as Fig. 1, but showing the values obtained with young virgin female rats.

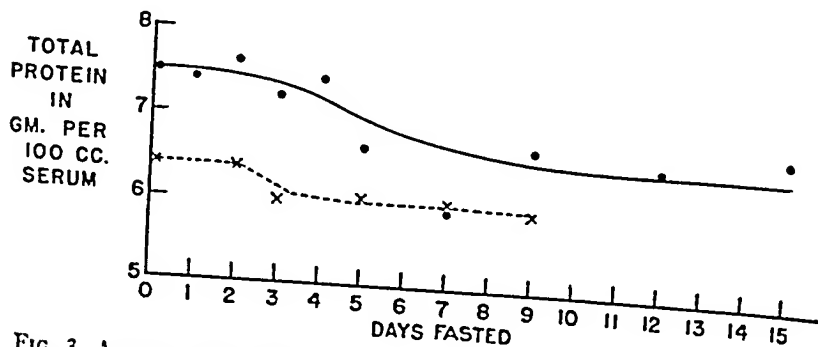


FIG. 3. Average values for the serum protein concentration. Each value on the chart represents the average of the corresponding individual values as shown in Figs. 1 and 2. Dots and solid line represent the values in the mature animals; crosses and broken line those of the young rats.

A glance at Figs. 1 and 2, however, makes clear at once that there are marked individual variations from the trends shown in the aver-

ages. In the old rats, for example, individuals maintain levels above 7.5 gm. of total protein per 100 cc. of serum for as long as 9 days of

TABLE I

*Summary of Averaged Values Obtained in Observations on the Series of Young Rats*

| Experimental group | Original weight | Weight when killed | Per cent of original weight when killed | Blood volume | Total protein   |
|--------------------|-----------------|--------------------|---|--------------|-----------------|
|                    | gm.             | gm.                |   | cc.          | gm. per 100 cc. |
| Controls           | 128.8           | 128.8              | 100.0                                   | 4.6          | 6.40            |
| Fasted 2 days      | 126.8           | 114.2              | 90.0                                    | 3.9          | 6.38            |
| " 3 "              | 116.8           | 102.8              | 88.0                                    | 3.9          | 6.02            |
| " 5 "              | 120.4           | 95.4               | 79.2                                    | —            | 6.07            |
| " 7 "              | 124.4           | 93.6               | 75.2                                    | 3.1          | 6.01            |
| " 9 "              | 118.4           | 80.0               | 67.6                                    | 2.4          | 5.94            |

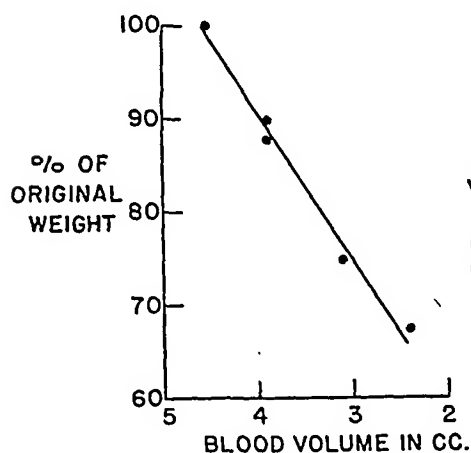


FIG. 4a

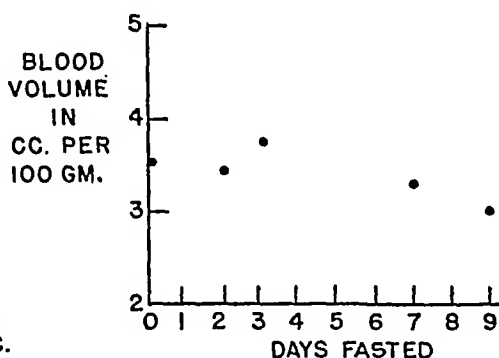


FIG. 4b

FIG. 4a. The relation of blood volume to weight loss. The values plotted are averages for all the animals in each group, as shown in Table I. Blood volume is to be understood as that quantity of blood obtained by exsanguinating the animal as completely as possible from the great vessels of the abdomen, with no attempt to wash out final traces of blood.

FIG. 4b. The plotted values are averages of the individual groups, as in Fig. 4a.

fasting. This individual resistance seems of theoretical importance and will be referred to again.

Figs. 4 *a* and 4 *b* show the relationship between body weight and blood volume as measured by exsanguination. As is well known, the method of determining the total volume of blood by simply exsanguinating the animal is not entirely accurate. The relationships shown in the charts, however, appear so clear that it was felt that the results at least approximated the true state of affairs, and the data are therefore presented. Although the blood volume is directly re-

TABLE II

*Details of Some of the Experiments on Individual Rats of the Mature Series, to Show Lack of Correlation between Body Weight and Serum Protein Concentration*

| Weight of rat | Total serum protein | Weight of rat | Total serum protein |
|---------------|---------------------|---------------|---------------------|
| gm.           | gm. per 100 cc.     | gm.           | gm. per 100 cc.     |
| Controls      |                     | Fasted 3 days |                     |
| 244           | 7.74                | 246           | 7.36                |
| 236           | 7.26                | 220           | 7.54                |
| 224           | 7.42                | 218           | 7.15                |
| 218           | 7.96                | 204           | 6.93                |
| 192           | 7.25                | 202           | 7.13                |
| Fasted 5 days |                     | Fasted 9 days |                     |
| 258           | 6.70                | 202           | 5.96                |
| 242           | 7.39                | 200           | 7.75                |
| 220           | 6.67                | 194           | 7.32                |
| 206           | 6.97                | 194           | 6.56                |
| 196           | 7.38                | 188           | 5.95                |
| 158           | 5.78                | 184           | 6.69                |
| 152           | 7.68                | 182           | 6.81                |
| 150           | 6.66                | 162           | 7.02                |
| 146           | 6.24                | 158           | 6.74                |
| 140           | 5.77                | 134           | 6.26                |

lated to the weight of the body, the serum protein concentration shows no correlation whatever with size or weight. The figures from several of the experiments, presented in detail in Table II, show this.

It is fairly well established that the albumin fraction of the serum proteins is the more labile, and its concentration is altered more readily in various disease states than that of the globulin fraction, though this is by no means an invariable rule. However, from the results shown in Table III it seems clear that the fall of concentra-



tion of plasma protein in the fasting rat is due to a decrease in the albumin fraction with the globulin changed little or not at all.

### DISCUSSION

What light do these results throw on the hypothesis of a circulating fraction of protein utilizable by the tissues and used by the cells of the body in preference to the more resistant organized tissue protein, or in the words of the more modern champions of the same hypothesis, on the question of give and take between body protein and plasma protein?

At first glance it might seem that these experiments offer evidence in favor of such a hypothesis. The definite decreases shown in Fig.

TABLE III

*Albumin-Globulin Ratios on Pooled Sera from Fasting Rats*

| Experimental group | No. of rats<br>in group | Total<br>protein           | Albumin                    | A/G ratio |
|--------------------|-------------------------|----------------------------|----------------------------|-----------|
|                    |                         | <i>gm. per<br/>100 cc.</i> | <i>gm. per<br/>100 cc.</i> |           |
| Controls           | 4                       | 6.90                       | 3.15                       | 0.84      |
| Fasted 6 days      | 4                       | 6.95                       | 2.00                       | 0.40      |
| " 10 "             | 3                       | 6.12                       | 2.50                       | 0.69      |
| " 14 "             | 4                       | 6.14                       | 2.25                       | 0.58      |

3 might be interpreted as due to the sudden giving up of some special fraction of labile protein to the tissues following exhaustion of their own reserves. A study of the results in individual cases, however, shows that this explanation is not the probable one. Although in the old rats the average drop occurs at about the 5th day, some individuals maintain a normal concentration of plasma protein for as long as 9 days. It is difficult to conceive of these individuals as having sufficient nitrogenous reserves somewhere in their tissues to last 9 days before it becomes necessary to call on an easily available protein constantly at hand in the blood stream.

The relationship of the blood volume to weight in the fasting animals also appears to be pertinent. It is clear that as body weight decreases the blood also decreases. This can only mean that a

certain portion of all of the blood proteins is destroyed to provide fuel and nitrogen for essential vital tissues. The concentration of proteins in the diminished volume of serum is not increased, but on the contrary shows the early decreases noted and then maintains itself stubbornly for long periods.

It would seem more logical to suppose that the early drop in the more labile albumin fraction of the blood plasma represents the same destruction that results in decrease in size of any tissue during fasting, and that the later maintenance of the concentration of the plasma proteins is a part of this same conservation of tissue whose function is essential to life. Physiologists have long been familiar with this conservation of tissue in the case of such organs as heart and brain, which maintain their integrity at the expense of muscle and other less vital tissue. From the point of view of normal function, the plasma protein is assuredly an essential tissue.

No evidence is at hand regarding the process by which the concentration of protein in the plasma is maintained following the initial drop. It appears from the present experiments that age is one factor. Mature rats maintain the plasma proteins somewhat more tenaciously than young, active growing animals. Another very definite factor is that complex of unknown characteristics which is summarized as individual resistance. Beyond this one cannot at present go.

#### SUMMARY AND CONCLUSIONS

1. Experiments were carried out to test the hypothesis that there exists a special circulating fraction of plasma protein available for use by the tissues.
2. The changes in serum protein concentration after varying periods of fasting were followed in large numbers of individual rats.
3. Previous reports from this laboratory of a small initial drop in the total protein concentration of the serum, with subsequent maintenance of the serum protein at the new level are confirmed.
4. Evidence is offered that this initial fall involves chiefly or solely the albumin fraction.
5. The mechanism responsible for the observed initial drop and subsequent maintenance of the protein is not exactly known, but two definite factors are age and individual resistance.

6. It is concluded that no satisfactory evidence is available to support the hypothesis of a directly utilizable protein fraction in the blood.

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# THE IMMUNOLOGICAL SPECIFICITY OF STAPHYLOCOCCI

## I. THE OCCURRENCE OF SEROLOGICAL TYPES\*

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(Received for publication, April 1, 1935)

During the course of studies on the occurrence of heightened skin sensitivity to bacterial derivatives in patients with trachoma (1), reactions were observed that were reconcilable only on the predication that staphylococci are composed of immunologically different strains. Since the available information on this subject failed to furnish conclusive evidence that such a condition exists, it was decided to investigate the possible classification of these organisms into serologically distinct types. While a preliminary report (2) has already been made of the more salient aspects of this study, it is desirable to submit at the present time, the more detailed data and experiments.

### Methods

*Strains of Staphylococci.*—The organisms studied in this investigation were isolated from a variety of sources during the past 2 to 3 years. The majority of the strains were cultivated from patients in Barnes Hospital (St. Louis) who were suffering from septicemia, osteomyelitis, furunculosis, acute conjunctivitis and, in one instance, pneumonia. These were considered as pathogenic, virulent strains. Other cultures were isolated from the normal conjunctiva and skin, from monkeys as bacteria fortuitously present in other infections, from the air, etc. Three cultures, Ha, Fs and D<sub>1</sub>, were obtained for purposes of comparison.<sup>1</sup> The first two of these strains are pathogenic and toxigenic, while the last is both avirulent and non-toxicogenic (3).

*Immunization.*—Only normal rabbits not possessing normal agglutinins for *Staphylococcus* were used for immunization. Broth cultures 15 to 18 hours old were centrifugated and the sediment was resuspended in sufficient saline to equal one-tenth the original volume. The bacteria were then killed by heating for 1 hour

\* Conducted under a grant from the Commonwealth Fund of New York.

<sup>1</sup> These cultures were kindly sent by Dr. E. L. Burky of Johns Hopkins Hospital.

at 56–60°C. In making injections the suspensions were diluted to original volume. The animals were given four courses of intravenous injections consisting of inoculations on 3 successive days and a rest of 4 days. The dosage for the first course was 0.5 cc., and this was increased 0.5 cc. each course, until 2.0 cc. were injected. The animals were then bled 10 days after the last inoculation.

*Agglutination.*—Agglutinations were conducted with either living or heat-killed broth cultures. The usual technique was employed of incubating the tests in the water bath at 37°C. for 2 hours and storing in the ice chest overnight. Final readings were made on the following morning.

*Precipitation.*—Precipitation tests were conducted in the usual manner using 0.5 cc. of diluted antigen and 0.5 cc. of serum diluted 2:3.

### *Differentiation of Types by Precipitation*

Rabbits were immunized as described to eight different strains of *Staphylococcus*. Eventually all the antisera were tested for agglutination with 30 odd strains. For purposes of illustration, however, typical agglutination of 12 strains in four antisera are presented in Table I. Examination of this protocol reveals that none of the cultures were agglutinated in sera pooled from six normal rabbits. It is important in this connection to point out that agglutination of *Staphylococcus* is not uncommon in normal rabbit serum. Further observation of Table I indicates that with some of the more recently isolated strains (13, C, 158) there appears to be a distinct tendency to separate into different immunological entities. On the other hand, in the case of some other strains (as B<sub>2</sub>A, and D<sub>1</sub>) a difference in serological reactivity is suggestive only, while in the remaining agglutinations (e.g. K, P, 161, Mx3), there is no indication of the existence of different types. In fact an analysis of all the agglutination reactions observed in this study offers at best only a suggestion of type differentiation, and the conclusion is therefore unavoidable that agglutination presents an uncertain and inconclusive method for distinguishing immunological types among the staphylococci.

### *Differentiation of Types by Precipitation*

The original observation by Avery and Heidelberger (4) that the type specificity of pneumococci is determined by chemically different carbohydrates or soluble specific substances has been confirmed and enlarged upon by studies of other bacteria as well as *Pneumococcus*. The results of these combined studies indicate that in the present

TABLE I  
*Representative Cross-Agglutination Reactions with Different Strains of Staphylococcus*

[illegible]

state of knowledge immunological specificity of bacteria is most frequently a property associated with the polysaccharide fraction of the cell. Since, as pointed out above, strains of staphylococci occasionally exhibit a definite tendency to type specificity it seemed wise to investigate the occurrence and behavior of carbohydrates derivable from these organisms. Ultimately a satisfactory method for extracting polysaccharides from staphylococci was devised. This method together with the chemical properties and characteristics of the soluble specific substances will be the subject of a subsequent report.

Preliminary precipitation tests with the soluble specific substances of different strains indicated in certain instances a serological reactivity in dilutions up to 1 to 6 to 8 million. It was soon discovered, however, that while all antibacterial sera contain agglutinins in high titre, anticarbohydrate antibody is frequently lacking, or present in titres too low for utilization in a study of differential precipitation. Since the sera of animals receiving the same suspensions of bacteria vary in the presence of precipitins, it must be assumed that the absence of antibody formation is dictated by the individual rabbit rather than the antigenic carbohydrate complex. From the data already on hand, it appears that only one of three or four antisera contain precipitins for the homologous polysaccharide. This figure, however, must be accepted as tentative, since the number of sera studied is not sufficiently extensive to allow accurate generalization.

Precipitation tests have been made with a number of purified carbohydrates derived from different strains of *Staphylococcus*. In Table II typical reactions of the polysaccharides from 12 different strains are presented. The results of these reactions show that on the basis of precipitation of the purified carbohydrates in antibacterial sera, staphylococci are sharply separable into two different and distinct types. As a matter of fact, a total of 16 strains have been studied by this method, and they all fall into one or the other of the two types. Of the cultures studied, nine isolated from different human infections and therefore considered virulent have fallen into one type, while the remaining, all isolated from non-pyogenic sources and consequently regarded as avirulent, have fallen into the second type. It is proposed, therefore, to designate the pathogenic strains as Type A and the non-pathogenic as Type B.

TABLE II

Precipitation of Purified Carbohydrates Derived from *Staphylococcus* in Homologous and Heterologous Antibacterial Sera

| Antisera and dilutions of carbohydrates expressed in thousands |     | Anti-B <sub>2</sub> A |     |     |     |     |      |          |     |     |     |     |      | Anti-Mx3 |     |     |     |     |      |
|--|-----|-----------------------|-----|-----|-----|-----|------|----------|-----|-----|-----|-----|------|----------|-----|-----|-----|-----|------|
| S.S. strain  | No. | Anti-13               |     |     |     |     |      | Anti-148 |     |     |     |     |      | Anti-Mx3 |     |     |     |     |      |
|  |     | 1:20                  | 50  | 100 | 200 | 500 | 1000 | 1:20     | 50  | 100 | 200 | 500 | 1000 | 1:20     | 50  | 100 | 200 | 500 | 1000 |
| 13   |     | +++                   | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| B <sub>2</sub> A   |     | +++                   | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| K  |     | +++                   | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| C  |     | +++                   | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| P  |     | +++                   | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| H <sub>2</sub> A   |     | +++                   | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| 148  |     | ---                   | --- | --- | --- | --- | ---  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| Mx3  |     | ---                   | --- | --- | --- | --- | ---  | ---      | --- | --- | --- | --- | ---  | +++      | +++ | +++ | +++ | +++ | +++  |
| Mx2  |     | ---                   | --- | --- | --- | --- | ---  | ---      | --- | --- | --- | --- | ---  | +++      | +++ | +++ | +++ | +++ | +++  |
| M31  |     | ---                   | --- | --- | --- | --- | ---  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| M <sub>2</sub>   |     | ---                   | --- | --- | --- | --- | ---  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |
| D <sub>1</sub>   |     | ---                   | --- | --- | --- | --- | ---  | +++      | +++ | +++ | +++ | +++ | +++  | +++      | +++ | +++ | +++ | +++ | +++  |

+++ heavy, compact, disk precipitate; +++ marked disk precipitate; ++ thin, film-like scale; + ground glass turbidity; ± slight turbidity.

All the carbohydrates indicated above were tested in the same dilutions in the pooled sera of six normal rabbits. Precipitations occurred in no instance.



That the carbohydrates are characteristic of *Staphylococcus* was later demonstrated by testing their reactivity in antibacterial sera of different species. The results of this experiment are given in Table III. Examination of this protocol discloses that while the carbohydrates of Type A and Type B *Staphylococcus* are precipitated in homologous immune sera, they do not precipitate in antipneumococcus, Type I, II or III sera, or anti-Friedländer, Type A, B or C sera, or in antityphoid serum. It is obvious, therefore, that the carbohydrates are genuinely specific of *Staphylococcus*.

The method of obtaining a relatively purified carbohydrate from *Staphylococcus* makes a rapid typing difficult. It was proposed, therefore, to determine the possibility of employing crude extracts of the bacteria for typing purposes. Accordingly, cultures were seeded into flasks containing 50 cc. of broth and they were incubated for 20 hours. The broth cultures were then centrifugated, and the clear supernatant fluid was saved, while the sedimented bacteria were extracted with acid. 10 cc. of 16/N HCl was added to the bacteria and the suspension was boiled in a water bath for 15 minutes. It was then cooled and made neutral to litmus by adding 16/N NaOH. This was then brought to original volume with saline and centrifugated. The clear supernatant was used in the precipitation test. The results of this experiment are recorded in Table IV. It will be seen that type specificity may be detected by precipitation tests performed with the supernatant of broth cultures. This is particularly true of Type A strains which apparently elaborate greater quantities of soluble specific substance. The reactions are even more striking when acid extracts are used for precipitation. In some instances it will be noted that there is a certain degree of cross-precipitation (*e.g.* Mx3) but when the reaction is specific the precipitate forms as a firm compact disk which is disrupted with difficulty, while the non-specific precipitate is granular and is disturbed readily on agitation. It appears, therefore, that with certain strains sufficient carbohydrate may be obtained for precipitation tests either by centrifugation of young broth cultures or by acid extraction of the sedimented bacteria.

Representative strains of both Types A and B were tested in rabbits for virulence and for toxicity of broth culture filtrates. The strains studied were 13, Ha and F<sub>s</sub> (Type A) and Mx3 and D<sub>1</sub> (Type

TABLE III  
Precipitation of Purified Carbohydrates Derived from *Staphylococcus* in Antisera of Different Bacteria

| Antiserum                           | Dilution of carbohydrate |       |       |       |     |      |  |              |     |     |     |     |      |  |
|-------------------------------------|--------------------------|-------|-------|-------|-----|------|--|--------------|-----|-----|-----|-----|------|--|
|                                     | Type A (13)              |       |       |       |     |      |  | Type B (Mx3) |     |     |     |     |      |  |
|                                     | 1:20                     | 50    | 100   | 200   | 500 | 1000 |  | 1:20         | 50  | 100 | 200 | 500 | 1000 |  |
| <i>Staphylococcus</i> , Type A..... | +++++                    | +++++ | +++++ | +++++ | +++ | ++   |  | +++          | +++ | +++ | +++ | ++  | +    |  |
| " " B.....                          | -                        | -     | -     | -     | -   | -    |  | -            | -   | -   | -   | -   | -    |  |
| <i>Pneumococcus</i> " I.....        | -                        | -     | -     | -     | -   | -    |  | +++          | +++ | +++ | +++ | ++  | +    |  |
| " " II.....                         | -                        | -     | -     | -     | -   | -    |  | -            | -   | -   | -   | -   | -    |  |
| " " III.....                        | -                        | -     | -     | -     | -   | -    |  | -            | -   | -   | -   | -   | -    |  |
| <i>Friedländer</i> " A.....         | -                        | -     | -     | -     | -   | -    |  | -            | -   | -   | -   | -   | -    |  |
| " " B.....                          | -                        | -     | -     | -     | -   | -    |  | -            | -   | -   | -   | -   | -    |  |
| " " C.....                          | -                        | -     | -     | -     | -   | -    |  | -            | -   | -   | -   | -   | -    |  |
| <i>Typhoid</i> .....                | -                        | -     | -     | -     | -   | -    |  | -            | -   | -   | -   | -   | -    |  |

TABLE IV  
*Precipitation of Crude Preparations of Carbohydrates in Homologous and Heterologous Antisera*

| Antigen                          | Dilution of antigen in |                  |                  |                  |                  |                  |                  |    |     |     |
|----------------------------------|------------------------|------------------|------------------|------------------|------------------|------------------|------------------|----|-----|-----|
|                                  | Type A serum           |                  |                  |                  |                  | Type B serum     |                  |    |     |     |
|                                  | 1:1                    | 5                | 20               | 100              | 200              | 1:1              | 5                | 20 | 100 | 200 |
| Supernatant 20 hr. broth culture |                        |                  |                  |                  |                  |                  |                  |    |     |     |
| 13 (Type A).....                 | +++ <sup>s</sup>       | +++ <sup>s</sup> | +++ <sup>s</sup> | +++ <sup>s</sup> | ++               | -                | -                | -  | -   | -   |
| B <sub>2</sub> A " ".....        | +++ <sup>s</sup>       | +++ <sup>s</sup> | +++ <sup>s</sup> | ++               | -                | ±                | -                | -  | -   | -   |
| Ha " ".....                      | +++ <sup>s</sup>       | +++ <sup>s</sup> | +++ <sup>s</sup> | +                | -                | -                | -                | -  | -   | -   |
| F <sub>8</sub> " ".....          | +++ <sup>s</sup>       | +++ <sup>s</sup> | +++ <sup>s</sup> | -                | -                | +                | -                | -  | -   | -   |
| Mx3 (Type B).....                | ++ <sup>g</sup>        | +                | ±                | -                | -                | +++ <sup>s</sup> | +++ <sup>s</sup> | +  | ±   | -   |
| D <sub>1</sub> " ".....          | -                      | -                | -                | -                | -                | +++ <sup>s</sup> | ±                | -  | -   | -   |
| 148.....                         | + <sup>g</sup>         | ±                | -                | -                | -                | +++ <sup>s</sup> | +++ <sup>s</sup> | +  | -   | -   |
| Neutralized acid extract         |                        |                  |                  |                  |                  |                  |                  |    |     |     |
| 13 (Type A).....                 | +++ <sup>s</sup>       | +++ <sup>s</sup> | +++ <sup>s</sup> | +++ <sup>s</sup> | +++ <sup>s</sup> | +                | ±                | -  | -   | -   |
| B <sub>2</sub> A " ".....        | +++ <sup>s</sup>       | +++ <sup>s</sup> | +++ <sup>s</sup> | ++               | +                | -                | -                | -  | -   | -   |
| Ha " ".....                      | +++ <sup>s</sup>       | +++ <sup>s</sup> | +++ <sup>s</sup> | +                | -                | ++               | ±                | -  | -   | -   |
| F <sub>8</sub> " ".....          | +++ <sup>s</sup>       | +++ <sup>s</sup> | +++ <sup>s</sup> | +++ <sup>s</sup> | +                | ++               | ±                | -  | -   | -   |
| Mx3 (Type B).....                | ++ <sup>g</sup>        | +                | ±                | -                | -                | +++ <sup>s</sup> | +++ <sup>s</sup> | ++ | ±   | -   |
| D <sub>1</sub> " ".....          | -                      | -                | -                | -                | -                | -                | -                | -  | ±   | -   |
| 148.....                         | +                      | -                | -                | -                | -                | ++               | ++               | ±  | -   | -   |

<sup>s</sup> indicates precipitate was of the compact disk variety; <sup>g</sup>, of the granular type readily disrupted on agitation.

B), and filtrates of these organisms were prepared according to the technique described by Burky (3). Strains 13 and Ha killed rabbits regularly, while  $F_s$  was irregular. Neither Mx3 nor  $D_1$ , however, when injected in similar quantities were virulent for rabbits. In testing the toxigenic properties of the same strains by the injection of broth filtrates in rabbits, it was found that filtrates of 13 and Ha were toxic while that of  $F_s$  was only slightly so. Neither filtrate of the Type B cultures studied caused any reaction in rabbits. It may be concluded, therefore, that Type A strains may be both virulent and toxigenic for rabbits but that Type B strains exhibit neither property. An attempt was made later to differentiate the two types of *Staphylococcus* on the basis of lysis by bacteriophage. Three strains of *staphylococcus* phage were obtained from Dr. J. J. Bronfenbrenner. The activity of the phage was then tested against representative strains of each type in the usually prescribed manner. The results of this experiment were definite in demonstrating that the function of lysis of *Staphylococcus* by bacteriophage is not related to the type of a given strain.

The small number of antisera containing type specific precipitins following intravenous immunization made it desirable to determine the effect of different methods of immunization on the formation of precipitins. Rabbits given repeated injections of heat-killed *staphylococci* intracutaneously showed high titres of agglutinins in their sera but in no instance antibodies reactive with the carbohydrates. In other rabbits, living organisms were injected in agar foci also intracutaneously, and while the agglutinin titre following this immunization was high, the sera was completely devoid of type specific precipitins. It may be concluded, therefore, that of the three methods studied, intravenous immunization is the most effective for stimulating the anticarbohydrate antibody.

#### DISCUSSION

That *staphylococci* are separable into different immunological types has been suggested by Julianelle (5) and Hine (6), on the basis of the agglutination reaction. So, also, Hopkins and Barrie (7) indicate a possible antigenic differentiation of this species as brought out by a modified agglutinin adsorption test. In each instance, however, the

types were not strictly specific nor did they show a sharp demarcation from each other. Similarly it has been observed in the present investigation that the agglutination reaction does not suffice in detecting types decisively. While more recently isolated strains have a distinct tendency to divide into different types, in many instances there is no indication even of type specificity. It was for this reason that a study was made to determine the specificity of carbohydrates derived from *Staphylococcus*.

The results of the experiments on the specificity of the carbohydrates reveal that at least two immunological types exist among the staphylococci. The indications are that pathogenic strains fall into one type, A, and the non-pathogenic strains comprise a second type, B. It is interesting to point out that both virulent and avirulent strains are capable of elaborating type specific carbohydrates and that virulence is therefore associated not with the presence of soluble specific substance, but rather with the particular type of polysaccharide. This it will be remembered is in contrast with *Pneumococcus* (8) and Friedländer's bacillus (9) where virulence accompanies elaboration of the type specific carbohydrate.

The differentiation of staphylococcus types may be demonstrated even with crude preparations of carbohydrates. Supernatant fluid of centrifugated broth cultures and acid extracts of the sedimented bacteria may also precipitate in homologous immune sera. The evidence indicates that the carbohydrates are apparently contained within the cell in striking contrast to the ectoplasmic distribution of the type specific polysaccharides of encapsulated bacteria. Attempts to distinguish type specificity on the basis of lysis by bacteriophage were unsuccessful.

The presence of type specific antibodies in the sera of immunized rabbits varies in individual animals despite a constantly high agglutinin titre in all antisera. The effect of different methods of immunization on precipitin formation was studied, and it was found that type specific precipitins are stimulated only following intravenous injections of the bacteria.

#### SUMMARY AND CONCLUSIONS

1. Agglutination is not a precise method for the demonstration of serological types among staphylococci.

2. Precipitation of soluble specific substance derived from these organisms demonstrates the existence of at least two immunologically distinct types.
3. The one type, designated A, is composed of apparently virulent strains, while the other, Type B, contains the avirulent strains.
4. Precipitation tests performed with centrifugates of young broth cultures or with acid extracts of sedimented bacteria may also demonstrate type specificity.
5. Lysis by bacteriophage fails to detect the specific types of *Staphylococcus*.
6. Immunization by intravenous methods stimulates agglutinin formation in all rabbits and precipitin formation in only one of three or four animals.
7. Immunization by repeated intracutaneous injections of dead staphylococci or living organisms in an agar focus also stimulates agglutinin formation but fails to incite the formation of type specific precipitins.

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# THE IMMUNOLOGICAL SPECIFICITY OF STAPHYLOCOCCI

## II. THE CHEMICAL NATURE OF THE SOLUBLE SPECIFIC SUBSTANCES\*

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The evidence presented in a previous communication (1) indicates the occurrence of at least two specific types among the staphylococci. While the specificity of the types, however, is occasionally detected by the agglutination reaction, it is demonstrable regularly only by precipitation in homologous antisera, of the soluble specific substances extracted from the organisms. In the preparation of the reactive material a number of methods have been tried, and in a general way, all have been more or less successful with the results varying in quantity of material recovered. It is proposed in the present report to describe the method of extraction and purification finally adopted and to present with this method the chemical properties and characteristics of the reactive substances.

While purified soluble specific substance has been isolated from several different strains of *Staphylococcus*, the bulk and detail of the study has been conducted with Strains 13 and Mx3. The former is now identified as Type A and was originally isolated from a patient with septicemia. The latter is a Type B strain and it was cultivated from the normal conjunctiva.

### *Isolation of the Soluble Specific Substances*

The bacteria centrifugated in a Sharples from 50 liter lots of 18 to 24 hour broth cultures of staphylococci were suspended in approximately 200 cc. of N/16 hydrochloric acid. Such suspensions were kept in the ice box until the bacteria from 250 liters were accumulated. The suspensions were then extracted by heating in a boiling water bath for 20 minutes. When the mixture had cooled sufficiently to be transferred to 250 cc. bottles it was centrifugated and the clear

\* Conducted under a grant from the Commonwealth Fund of New York.



straw-colored supernatant was decanted. At this point a great deal of the inactive material was removed by dropwise addition of 40 per cent alkali (NaOH). The resulting flocculent precipitate was then removed by centrifugation. The supernatant was precipitated again with the strong alkali and separated from the resulting precipitate. This process was repeated until no precipitation resulted from the further addition of alkali. At times, after most of the protein had been precipitated the solution became hazy when more alkali was added, but without the formation of a precipitate. On such occasions, the solution was allowed to stand overnight in the ice box, after which time the coagulated protein was removed.

The clear supernatant (acid to litmus) was treated with two to four volumes of alcohol and about 5 gm. of sodium acetate. The partially precipitated solution was allowed to stand in the ice box overnight. By the next morning a heavy precipitate had settled to the bottom of the flask and most of the alcoholic supernatant was removed by decantation. The precipitate was completely separated from the supernatant by centrifugation. The residue was extracted with 50 to 100 cc. of water (depending on the quantity of precipitate) and any insoluble material was removed by centrifugation. The aqueous extract was precipitated with a solution of 50 per cent trichloroacetic acid. After removal of the precipitated material the supernatant was again precipitated with trichloroacetic acid and centrifuged. This process was repeated until no precipitation followed the addition of trichloroacetic acid. The resulting supernatant was then precipitated with six volumes of alcohol. The very turbid solution was allowed to stand for several days in the refrigerator, after which time the clear alcoholic supernatant was completely decanted from the gummy precipitate which adhered to the walls of the flask. After the last traces of alcohol had been separated from the gummy precipitate by suction it was extracted with a minimum quantity of water (10 to 25 cc.). Any undissolved material was removed and the supernatant was precipitated with trichloroacetic acid as described above. The processes of precipitation with alcohol, extraction of the gummy residue with water and subsequent precipitation of this extract with trichloroacetic acid were repeated until the aqueous extract no longer contained material precipitable with trichloroacetic acid.

The resulting aqueous extract was then diluted to about 100 cc. and decolorized with norit. The clear filtrate was electrodialed in a cell similar to that described by Holmes and Elder (2). From time to time a drop or two of concentrated hydrochloric acid was added to the cell and the dialysis was continued until the ammeter reading had remained constant for at least 2 hours after the preceding addition of hydrochloric acid. The final ammeter reading varied from 0.005 to 0.008 amperes. The dialysate was concentrated *in vacuo* to about 10 to 15 cc. and then poured into ten volumes of dry redistilled acetone. After the turbid solution had stood in the refrigerator overnight the precipitated material settled to the bottom of the flask. The clear supernatant was poured off and the gummy precipitate was triturated with dry acetone until it became sufficiently hard and dry to permit effective centrifugation. The now granular precipitate was washed three or four times with absolute alcohol and finally with ether. The yield varied

from about 0.025 to 0.05 gm. This yield does not include, however, the material extracted from waste residues and supernatants which were extracted separately.

### *Chemical Properties of the Soluble Specific Substances*

The soluble specific substances of both Types A and B obtained by the procedure described above are white amorphous powders, readily soluble in water, giving crystal clear aqueous solutions in concentrations of 1 per cent. The substances possess acid properties, since aqueous solutions give an acid reaction to litmus. The Molisch reaction is positive in high dilutions and Fehling's solution is not reduced. When the substances are boiled with mineral acid, however, they are slowly hydrolyzed with the formation of reducing sugars which have not been identified completely as yet. With the chemical change accompanying hydrolysis, the soluble specific substances simultaneously lose their serological reactivity. The substances give no color with iodine-potassium iodide solution. Dilute solutions (1:200) give yellow crystals of iodoform with sodium hypiodite.

1 per cent solutions of the soluble specific substances do not give the usual color or precipitation reactions characteristic of proteins. Such tests as Millon's, xanthoproteic, Hopkins-Cole and ninhydrin tests are negative. The biuret reaction also may be considered negative, since the color and intensity of the resulting solution match those of a dilute solution of egg albumin (1:2000), which is about the limit of delicacy of the test. Solutions of the substances are not precipitated by the salts of heavy metals (e.g. mercuric chloride, silver nitrate or neutral lead acetate), by acids (e.g. trichloroacetic acid, picric acid, sulfosalicylic acid and potassium ferrocyanide and acetic acid) or by concentrated solutions of salts such as ammonium sulfate, sodium sulfate, sodium chloride, etc. Uranyl nitrate does not cause immediate precipitation but if the solution is allowed to stand for several days a yellow precipitate is obtained.

While the Type A and Type B soluble specific substances probably do not represent single, chemically pure compounds, it seems, nevertheless, that the major portion of the impurities have been removed. On the basis of the properties described above, it is reasonable to conclude that the serum reactive substances are polysaccharides. The chemical data bearing on the nature of both soluble specific substances are recorded in Table I and they indicate the constancy of composition of various preparations.

In studying the properties submitted in Table I, it is obvious that some of the data merit comment. The values for carbon are low since the theoretical value for a polysaccharide of formula  $(C_6H_{10}O_5)_x$  is 44 per cent. The consistently high percentage of phosphorus which appears to be due neither to inorganic phosphate nor protein

(as shown above) suggests the possibility that the polysaccharides may be phosphoric acid derivatives. In this connection it is interesting to note that the calculated values for carbon and phosphorus for a monophosphate of a disaccharide are 33 and 7 per cent respectively. It is possible that the polysaccharides may be complexes in which the same ratio, of two simple hexose units to one of phosphoric acid, is present. Although the polysaccharides are apparently protein-free it has not been possible as yet to isolate a preparation

TABLE I

*Chemical Characteristics of the Specific Carbohydrates of Types A and B Staphylococcus*

|  | Type A* |        | Type B* |        |
|--|---------|--------|---------|--------|
|  |         |        |         |        |
| Ash, per cent.....                       | 1.8     |        | 3.5     |        |
| Carbon,† per cent.....                   | 34.70   |        | 36.12   |        |
| Hydrogen, per cent.....                  | 6.41    |        | 6.58    |        |
| Nitrogen, per cent.....                  | 4.09    |        | 3.84    |        |
| Phosphorus, per cent.....                | 6.27    |        | 6.40    |        |
| Neutral equivalent N/50 NaOH.....        | 776     |        | 806     |        |
| Optical rotation $[\alpha]_D^{20}$ ..... | +6.7°   | +7.57° | +69.4°  | +65.4° |
| Total reducing sugar, ‡ per cent.....    | 26.1    | 24.0   | 38.84   | 36.7   |
| Fermentable sugar, per cent.....         | 1.78    | 1.4    | 35.01   | 33.3   |
| Non-fermentable sugars, per cent.....    | 24.32   | 22.6   | 3.87    | 3.4    |

\* Both substances reacted in homologous immune sera to a dilution of 1:6,000,000.

† All percentages are calculated on ash-free basis.

‡ Shaffer-Hartmann procedure used for determining the reducing sugars. All values are calculated as glucose.

completely free of nitrogen. Whether this nitrogen is an impurity or an integer of constitution as Avery, Goebel and Heidelberger (3) have shown for Type I Pneumococcus must await future work. The optical rotations of the two preparations are given for each type and as can be seen these values are almost identical; likewise, the quantity of reducing sugars are of the same order of magnitude. The similar chemical composition of the two polysaccharides is indeed as impressive as the difference in the serological reactivity. On the basis of the carbon, hydrogen, nitrogen and phosphorus content as well as the

neutral equivalents, it would seem that the two polysaccharides are empirically strikingly similar.

### *The Hydrolytic Products of the Soluble Specific Substances*

As a preliminary approach to a study of the hydrolytic products of the type polysaccharides, observations were made on the rotation between time of hydrolysis, alterations in optical rotation and loss in serological specificity of the carbohydrates. For this purpose, 0.2322 gm. of Type A and 0.2281 gm. of Type B polysaccharides were dissolved in 10 cc. of normal sulfuric acid. The optical rotations and precipitation in homologous immune sera were recorded, and the solutions were then hydrolyzed in sealed tubes in a boiling water

TABLE II

*Relation of Serological Specificity and Optical Rotation to Acid Hydrolysis*

| Period of boiling | Type A carbohydrate                   |                      | Type B carbohydrate                   |                      |
|-------------------|---------------------------------------|----------------------|---------------------------------------|----------------------|
|                   | Optical rotation<br>$[\alpha]_D^{25}$ | Serological activity | Optical rotation<br>$[\alpha]_D^{25}$ | Serological activity |
| 0                 | +6.7°                                 | ++++                 | +69.4°                                | ++++                 |
| 2 hrs.            | 19.1°                                 | +                    | 29.5°                                 | ++                   |
| 4 hrs.            | 19.6°                                 | —                    | 22.1°                                 | —                    |
| 5½ hrs.           | 21.2°*                                | —                    | 23.1°*                                | —                    |

\* If calculated on the basis of reducing sugars, the optical rotation for Type A = +81.1°, for Type B = +59.7°.

bath. At definite intervals, readings were made on the serological titres and optical rotations, the latter figures calculated on the basis of weight of polysaccharide. The results of this experiment are presented in Table II. Examination of this protocol reveals that serological specificity is greatly diminished within 2 hours, and that it is destroyed completely within 3 hours. Similarly, the alterations in optical rotation indicate an increasing hydrolysis up to about 5 hours when the polysaccharides appear to be totally hydrolyzed.

### *Preparation of Osazones from the Products of Hydrolysis*

The solutions from the hydrolyses described in the above paragraph were used for the preparation of the osazones. The solutions were

diluted to about 50 cc. and neutralized with barium carbonate. The barium sulfate was then filtered off. The filtrate was treated with a very small quantity of norit and an excess of calcium carbonate. After the mixture had been heated to boiling, it was filtered and the resulting filtrate was concentrated *in vacuo*. The residue was extracted three times with 10 cc. of methyl alcohol for each extraction. The alcoholic extracts were filtered and concentrated *in vacuo* and the residue was taken up in about 10 cc. of water. The aqueous extract was filtered and then treated with 0.25 gm. of a mixture consisting of two parts recrystallized phenylhydrazine hydrochloride and three parts of sodium acetate.

*Characteristics of Osazone Derived from Type A Carbohydrate.*—Immediately after the phenylhydrazine mixture had completely dissolved, the solution became hazy. After shaking, a yellow feathery precipitate began to settle out. The mixture was allowed to stand at room temperature for several hours after which it was filtered. An attempt was made to recrystallize the material but without success. The filtrate was heated in a water bath for 2 hours and the clear solution was allowed to cool very slowly. The crystals which had settled out were examined under the microscope, and they were found to consist of rosettes of ferny petals. The crystals were filtered off and washed with alcohol. The melting point of the crystals was 195°.

More phenylhydrazine acetate mixture was added to the filtrate and the resulting mixture was heated in the water bath. No more crystals were obtained.

A second portion of Type A was hydrolyzed and treated as above except that the yellow precipitate obtained on the addition of the phenylhydrazine mixture was not removed. The entire mixture was heated for 2 hours in the water bath. The same type of crystals were obtained with a melting point of 195°.

The information gained on the osazone derived from Type A polysaccharide fails to identify the simple sugar resulting from hydrolysis of the original carbohydrate.

*Characteristics of Osazone Derived from Type B Carbohydrate.*—The solution obtained by treating the simple sugar with phenylhydrazine acetate mixture was perfectly clear even after standing several hours at room temperature. After the mixture was heated

on the boiling water bath for an hour, the resulting crystals were filtered off. A second fraction was obtained by heating the solution further. The crystals were combined and recrystallized from 60 per cent ethyl alcohol. The melting point was  $204^{\circ}$ . A mixed melting point with glucosazone showed no depression.

The melting point and crystalline structure of the osazone from Type B are identical with those of glucosazone. The identification of glucosazone limits the sugar formed on hydrolysis of the specific substance to glucose or its amine, fructose or mannose. Since the hydrolyzed material does not give a Seliwanoff reaction and it does not yield an insoluble hydrazone, it appears likely that the simple sugar of Type B is glucose. Conclusive identification of glucose, however, is lacking at present, but with accumulation of material it is planned to oxidize the sugar to the corresponding acid thus making it possible to arrive at its identification through its potassium salt.

Differences in the nature of the simple sugars of the two specific carbohydrates are also brought out in the quantitative determination of the reducing sugars as will be seen on restudy of Table I. The Shaffer-Hartmann procedure was used for the determination of reducing sugars, and all values are calculated as glucose. It is interesting that the sugars resulting from hydrolysis of Type A polysaccharides are chiefly non-fermentable while those of Type B are fermentable. Since the phloroglucinol and orcinol reactions are negative it would seem that the non-fermentable sugar of Type A is not due to pentoses or uronic acids. Since qualitative tests are at their best extremely unreliable, no interpretation of the quantitative results is attempted at this time.

#### DISCUSSION

That the two soluble specific substances of *Staphylococcus* are immunologically distinct has been shown in a previous report (1). It was perhaps to be expected on the basis of different serological reactivity that the two substances were also chemically different. The observations reported in this communication give experimental verification of this assumption. While both substances have not been recovered free of nitrogen, it is, nevertheless, obvious that they are essentially carbohydrates and that their serological specificity disappears as hydrolysis of the soluble specific substance proceeds.

The chemical differences between Type A and Type B carbohydrates have been demonstrated in this report. The specific rotation of Type A is  $+6.7$  as compared with  $+69.4$  for Type B. Following hydrolysis, Type A yields 26.1 per cent reducing sugars of which 1.78 per cent is fermentable and 24.32 per cent is non-fermentable. Type B on the other hand yields 38.84 per cent reducing sugars of which 35.01 per cent is fermentable and 3.87 per cent is non-fermentable. Moreover, the simple sugar of Type B seems to be glucose while that of Type A remains undetermined but suggests, because of its crystalline formation, a different structure.

#### SUMMARY AND CONCLUSIONS

1. Two carbohydrates have been extracted from different strains of *Staphylococcus* which are immunologically and chemically distinct.
2. The chemical differences between the two types are manifested principally in optical rotation, and in the simple sugars resulting from hydrolytic cleavage of the specific carbohydrates.
3. The immunological specificity of both polysaccharides is dissipated as hydrolysis proceeds.
4. The results of the chemical reactions reported are discussed.

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# THE IMMUNOLOGICAL SPECIFICITY OF STAPHYLOCOCCI

## III. INTERRELATIONSHIPS OF CELL CONSTITUENTS\*

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That staphylococci may be classified into at least two immunological types has been reported in a previous communication (1). The separation into distinct types is determined by the elaboration of a specific carbohydrate which for strains derived from pathogenic conditions is chemically different from that produced by organisms isolated from saprophytic sources (2). While, therefore, precipitation of the polysaccharide extracted from different cultures exhibits a striking type specificity, agglutination of the bacteria, themselves, on the other hand, reveals a much broader or species specific reactivity. It was consequently suspected that the species specificity observed in the agglutination reaction might be governed by a common reactive protein constituent of the cell irrespective of type distinction. Since a similar condition has already been demonstrated in the case of *Pneumococcus* (3), and later Friedländer's bacillus (4) and *Streptococcus* (5), this possibility seemed sufficiently plausible to merit investigation. Accordingly a study has been undertaken of the interrelationships of the protein and carbohydrate derivatives of *Staphylococcus*, and the results of this study are reported at the present time.

### *Methods*

*The Specific Carbohydrates.*—Purified specific carbohydrates were prepared by the method described in a preceding communication (2). The technique of precipitation has also been outlined (1).

*The Protein Solutions.*—Protein solutions of representative strains of Type A and Type B *Staphylococcus* were prepared from young broth cultures (16 to 20 hours). The sedimented bacteria from 6 to 8 liters of culture were spread with a

\* Conducted under a grant from the Commonwealth Fund of New York.



spatula in a thin layer in a Petri dish. The organisms were then placed in the incubator (37°C.) for 30 to 60 minutes to allow more or less thorough drying. After desiccation, the organisms formed a somewhat sticky, gummy mass which made grinding difficult and lengthy so that the expedient of washing the dried bacteria first with alcohol and then with ether was resorted to, apparently without affecting the antigenicity or reactivity of the protein. The extracted and dried bacteria were then ground in a special grinding apparatus devised for the purpose in this laboratory (6). Gram stains of the bacterial mass were made from time to time to ascertain the degree of disintegration which, as a rule, was complete within 6 hours or less. The finely ground powder was then taken up in N/100 NaOH to effect solution of the protein. After centrifugation, the protein was precipitated with a minimum amount of normal acetic acid. The precipitate was collected and redissolved in alkali. Acid precipitation and solution with alkali were usually repeated two or three times and the final product was made up in saline made slightly alkaline to litmus. Solutions used for immunization were filtered through a Berkefeld V filter.

The method of immunization employed was that described in an earlier report (1).

### *Properties of the Specific Carbohydrate*

*Antigenic Properties.*—That chemically purified carbohydrates derived from bacteria exert no antigenic effect was originally believed by a number of investigators. More recently, scattered reports (7) indicated, however, that bacterial carbohydrates particularly in the case of *Pneumococcus* Type I, may serve as antigens under conditions which were not clearly understood. The confusion into which the subject had been thrown was eventually clarified by the observations of Avery and Goebel (8), who showed that in the case of *Pneumococcus* Type I at least, the polysaccharide exists as an acetylated carbohydrate which is antigenic, but that in the process of purification usually employed deacetylation occurs with a concomitant loss of antigenicity.

A study of the antigenicity of the polysaccharides of *Staphylococcus* indicated that in the condition originally used, they possessed no antigenic properties if the failure to stimulate agglutinins or precipitins in rabbits be accepted as a measure of antigenicity. Further attempts to determine whether the carbohydrates are true antigens were made by acetylating the polysaccharides. But this artificial alteration of the carbohydrates did not modify the original lack of antigenicity. It should be pointed out that even in the case of *Pneumococcus* the acetyl polysaccharide does not elicit antibody formation in rabbits.

The antigenicity mentioned above refers only to the ability to stimulate in white mice active immunity to infection.

Since the ability of the whole organism to induce type specific precipitins in rabbits is not great (1), an effort was made to ameliorate the conditions necessary for antibody formation. In one experiment, the intact bacteria were acetylated and then injected repeatedly intravenously but without inciting specific precipitin improvement in any of the rabbits studied. Adsorption of the soluble specific substance on collodion particles as described by Zozaya (9) was next investigated. By this method, also, the specific carbohydrates of *Staphylococcus* revealed no measurable antigenic effect.

*Serum Reactive Properties.*—That the soluble specific substances of *Staphylococcus* react to high titre in homologous immune serum, has already been reported (1). It is therefore obvious that the specific polysaccharides are true haptenes as originally defined by Landsteiner (10).

*Skin Reactive Properties.*—Since the study on the skin reactive properties of the carbohydrates of *Staphylococcus* is still under way, it is not desirable to make a complete report of cutaneous reactions at the present time. It will suffice, therefore, merely to point out that the polysaccharides may elicit skin reactions in patients recovering from *Staphylococcus* infection. As small quantities as 0.2 cc. of a 1:200,000 dilution may be sufficient to cause a type specific, wheal and erythema reaction. Thus far all the reactions have been to Type A carbohydrate only. Since, however, Type A strains alone appear to be pathogenic (1), it is to be expected on the basis of type specificity that Type B reactions will occur rarely, if at all. Experiments conducted in this laboratory (11) on the induction of skin reactivity in rabbits and monkeys indicate that skin reactions to the carbohydrates are elicited only rarely in these animals, but when they do occur they present the exquisite specificity usually observed in carbohydrate reactions. Thus, skin reactions to the polysaccharides may be observed under experimental conditions as well as during spontaneous infection in man.

#### *Properties of the Protein Constituent*

*Antigenic Properties.*—In contrast to the lack of antigenicity witnessed in the specific polysaccharides, the protein constituent of

*Staphylococcus* evokes an antibody response in rabbits. Antibodies are stimulated readily following intravenous or intracutaneous injections of the protein. The antibodies are demonstrable by precipitation. By the precipitation test, it may be observed that precipitins for the specific carbohydrates are not present in antiprotein sera. The precipitins for the protein, however, are present in relatively high titre. An examination of the data presented in Table I reveals in this connection that antiprotein sera precipitate protein derived not only from homologous strains, but protein derived from strains of heterologous type. In other words, unlike the carbohydrates, the protein constituent of *Staphylococcus* is an antigen devoid of type

TABLE I  
*Precipitation of Staphylococcus Protein in Antiprotein Sera*

| Antiprotein serum         | Protein*    | Dilution of protein |      |      |        |        |        |
|---------------------------|-------------|---------------------|------|------|--------|--------|--------|
|                           |             | 1:2400              | 4800 | 9600 | 19,200 | 38,400 | 76,800 |
| B <sub>2</sub> A (Type A) | 13 (Type A) | +++                 | +++± | ++   | +      | ±      | —      |
|                           | Mx3 ( " B)  | ++++                | +++  | +++  | ++     | +      | —      |
| Mx3 (Type B)              | 13 ( " A)   | +++                 | ++   | ++   | +      | —      | —      |
|                           | Mx3 ( " B)  | +++                 | +++  | ++   | +      | ±      | —      |

\* *Staphylococcus* protein does not precipitate in normal rabbit serum.

specificity and it is shared in common by all staphylococci irrespective of type distinction.

*Serum Reactive Properties.*—It has already been made obvious that the proteins derived from *Staphylococcus* as indicated react in antiprotein sera. That precipitation of the proteins occurs with equal readiness in antibacterial sera is brought out by the data given in Table II. It will be seen that proteins prepared from four different strains (two, Type A, and two, Type B) precipitate in both Type A and Type B antibacterial sera. While minor variations in titre are apparent, nevertheless the species specificity of the proteins is definitely manifested. The higher titre of precipitation in homologous immune sera may be explained on the basis that the proteins were not chemically pure and that they actually contained a certain amount of soluble specific substance which was also precipitated in the type

specific sera. This, then, may account for the apparently greater activity of the protein in homologous antisera.

*Skin Reactive Properties.*—Without attempting to make a final report now of the skin reactive properties of Staphylococcus protein, it is nevertheless desirable to indicate the contrast in skin reactivity of the protein and carbohydrate. As the serum reactivity suggests, the skin reactivity of the protein is also species specific. In addition, the skin reaction is not the immediate, wheal and erythema variety elicited by the carbohydrate, but the delayed inflammatory reaction

TABLE II  
*Precipitation of Staphylococcus Protein in Antibacterial Rabbit Sera*

| Protein from strain | Serum                | Dilution of protein* |     |     |    |     |     |
|---------------------|----------------------|----------------------|-----|-----|----|-----|-----|
|                     |                      | 1:10                 | 20  | 40  | 80 | 160 | 320 |
| 13, Type A          | Normal               | —                    | —   | —   | —  | —   | —   |
|                     | 13, Type A           | +++++                | +++ | ++± | ++ | +   | —   |
|                     | B <sub>2</sub> A " " | +++++                | +++ | ++  | +  | —   | —   |
|                     | Mx3 " B              | ++                   | ++  | +   | —  | —   | —   |
|                     | 148 " "              | +++                  | ++  | +   | ±  | —   | —   |
| Mx3, Type B         | Normal               | —                    | —   | —   | —  | —   | —   |
|                     | 13, Type A           | +++                  | ++  | +   | —  | —   | —   |
|                     | B <sub>2</sub> A " " | ++±                  | ++  | +   | ±  | —   | —   |
|                     | Mx3 " B              | +++++                | +++ | ++  | +  | ±   | —   |
|                     | 148 " "              | +++++                | ++± | +   | ±  | —   | —   |

\* The solutions of protein were used as made, without standardization, so that the dilutions are not necessarily comparable.

† Reactions in homologous sera were mixed precipitations of protein and carbohydrates.

frequently described as tuberculin-like. Experimentally, rabbits have been made regularly skin reactive to the protein of Staphylococcus, and it was observed that the reactions in these animals were species specific and delayed (11). The reactions were in every way similar to those occurring in man.

The skin reactive properties of both carbohydrates and proteins are still being studied in both normal individuals and in patients with Staphylococcus infection. The detailed experiments and statistical data, therefore, will necessarily await future work. The indications

are nevertheless definite on the nature of the specificity and the character of the reactions elicited by the two constituents. This will be recognized as paralleling the observations on the skin reactivity of the carbohydrates and nucleoprotein of *Pneumococcus* (12), which demonstrated for the first time the principles underlying skin reactions to these bacterial derivatives.

It may be of interest to report an additional experiment on the toxicity of the two products of *Staphylococcus*. Since toxigenic strains apparently fall in Type A, it was decided to determine the toxicity of the carbohydrates of each type. The results were definite in showing that neither Type A nor B carbohydrate is toxic for normal rabbits or white mice. Large quantities are tolerated with no evident effect even in the case of the Type A polysaccharide which was isolated from a known toxigenic strain. It may be concluded, therefore, that while the Type A carbohydrate may be present in toxigenic strains, it is not itself toxic. Similar experiments with the protein also indicate that this constituent possesses no toxic properties as determined in normal animals.

#### DISCUSSION

That the soluble specific substance of *Staphylococcus* determines the type specificity of the bacterial cell from which it is derived is a fact which has been confirmed by experiments described in this paper. In the form in which it exists in the cell, the specific carbohydrate is a poor antigen (1) as judged by its ability to stimulate specific antibody response in rabbits. When isolated in a state of high chemical purity, the soluble specific substances lose what weak antigenic properties they originally possess. Moreover, acetylation or adsorption of the polysaccharide on collodion particles does not affect measurably the lack of antigenicity. While the carbohydrate is highly reactive in sera, it precipitates only in antibacterial sera. In the skin of patients infected with *Staphylococcus*, the carbohydrate elicits an immediate wheal and erythema reaction which is specific to type.

The proteins, separated from the bacteria as described above, are species specific; that is, they are common to both types of *Staphylococcus*. They are antigenic in rabbits and they induce the formation of precipitins which react with protein solutions obtained from either

type. The proteins react both in antiprotein and antibacterial sera. In addition, they exhibit skin reactive properties which evoke the delayed, inflammatory reaction specific of the species rather than the type. That the protein of *Staphylococcus* is endowed with even a broader reactivity than that indicated in this report was pointed out by Lancefield (13) when she showed them to possess serological reactivities in common with *Streptococcus* and *Pneumococcus*.

#### CONCLUSIONS

1. The carbohydrates derived from *Staphylococcus* are type specific.
2. The specific carbohydrates fail to induce formation of antibodies in rabbits.
3. Acetylation or adsorption of the carbohydrates on collodion particles does not render them antigenic.
4. The specific carbohydrates may be employed to elicit immediate, type specific, skin reactions in patients with *Staphylococcus* infection.
5. The protein of *Staphylococcus* is species specific.
6. The protein is antigenic and stimulates in rabbits species specific antibodies.
7. The protein causes in hypersensitive individuals a species specific, delayed, inflammatory skin reaction.

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# THE DEGREE OF DISPERSION OF THE BACILLUS AS A FACTOR IN INFECTION AND RESISTANCE IN EXPERIMENTAL TUBERCULOSIS

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PLATES 1 TO 3

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Aqueous extracts of testicle contain a substance which produces a rapid and pronounced increase in the permeability of the dermis and connective tissues for injected fluids and suspensions (1-3). Toxins, suspensions of bacteria, and viruses may, with the aid of such a spreading agent, be dispersed through a greatly increased area of skin, and this dispersion brings about definite alterations in the nature of the resulting lesions (1, 4). In the case of toxins, and of antigens injected into previously sensitized animals, the dispersion of the material brings about a diminution of the intensity of the reaction, along with an increase in the area or extent of the lesion. The dispersion of large amounts of living bacteria augments both the size and severity of the lesion which develops, as compared with that produced by like numbers of bacteria injected in saline solution.

It was found (5) that when progressively smaller amounts of bacteria were injected together with extracts of testicle or of invasive staphylococci, the lesions were enhanced as a result of spreading, when their number in the suspension was above a minimal effective concentration; and were partially or totally suppressed when their number in the suspension fell below such a concentration. The virulence of the bacteria and the minimal effective concentration were found to be inversely related. Contrarily, the lesions produced by filterable viruses, even in quantities closely approaching the minimal infective dose, were enhanced by the dispersing factor.

The present paper deals with the effects of such dispersion on tuberculous infection in guinea pigs and rabbits. As in the case of other



bacteria, the extent of dispersion of injected tubercle bacilli is an important factor in the development of the primary infection as well as in the partial resistance elicited by reinfection, and in the resistance developed by vaccination with killed organisms.

The spreading agent used throughout this investigation was testicle extract. The fact that this extract acts by markedly increasing the permeability of the tissues has been proven in a series of studies (5-8). We have no evidence whatsoever that it has any direct action on any of the infectious agents themselves.

Walker and Hoffman (9) have already reported that intradermal injection into rabbits of testicle extract together with suspensions of human, bovine, and avian strains of tubercle bacilli resulted in more widespread lesions. Some of our own studies on the effect of testicle extract on tuberculous infection of guinea pigs have already been briefly reported (10).

### *Material and Methods*

#### *Characteristics of the Strains of Mycobacteria Employed*

*M. tuberculosis (Human Type, Strain H 37).*—This strain is of moderate virulence for rabbits when injected intravenously, producing a slowly progressive disease. Rabbits are markedly resistant to it when injection is made into or beneath the skin, practically never developing generalized lesions. Guinea pigs on the other hand are quite susceptible to infection with it, developing far advanced tuberculosis within 90 days after subcutaneous inoculation with 0.1 mg. or less.

*M. tuberculosis (Bovine Type, Strain B-1).*—This strain of bovine tubercle bacilli is but moderately virulent for rabbits, producing a very slowly progressive disease after intravenous inoculation.

*M. tuberculosis (Bovine Type, Strain Ravenel).*—This strain is highly virulent for rabbits, producing a rapidly progressive widespread disease after intravenous inoculation.

*M. tuberculosis (Avian Type, Strain Avian 1).*—This strain is avirulent for both rabbits and chickens at the present time, although 3 years ago it was highly virulent for both.

*B.C.G.*—This is the well known bacillus of Calmette and Guérin (used for prophylactic vaccination). It is avirulent for all laboratory animals. It was kindly supplied by the New York City Board of Health.

*Defatted Bovine Tubercle Bacilli.*—These organisms had been extracted with alcohol and ether for 2 months, following which they were dried and ground.

### *Preparation of Suspensions of Tubercle Bacilli*

Cultures grown on Corper's egg medium for 3 to 6 weeks were used. A suitable amount of organisms were harvested from the surface of the medium and weighed immediately. They were then triturated in a mortar with saline solution and finally brought to a concentration of 1.0 mg. of bacteria per cc. The addition of different volumes of saline solution to aliquot portions of the concentrated bacterial suspension provided a graded series of dilutions of bacteria. When heat-killed bacteria were employed they were prepared by immersing the flask containing the suspension in a water bath at 70°C. for 1 hour.

### *Preparation of the Spreading Agent*

Testicle extract was prepared from bull testicles. The membranes were stripped from the fresh organs, which were then minced and ground with sand and 4 volumes of 0.9 per cent saline solution. After centrifugation the extract was filtered through a Berkefeld V candle and then through a Berkefeld W candle. The extract was put into test tubes and stored in the refrigerator.

### *Method of Testing the Effect of Spreading of Tubercle Bacilli*

Mixtures were prepared generally containing 0.5 or 1.0 cc. of a bacterial suspension and testicle extract. These were injected into the skin of rabbits and guinea pigs. Similar suspensions containing saline solution instead of testicle extract were always injected as a control either into the same or another animal. When trying the effect of spreading different dilutions of bacillary suspensions in rabbits, no more than 5 injections were made on one side.

The injection of mixtures containing testicle extract was followed in both rabbits and guinea pigs by a marked spreading, as evidenced by the prompt flattening and disappearance of the wheal, and by the extent of the resulting edema and blanching.

Mature rabbits bred at The Rockefeller Institute from Lilac and English stock were used. Guinea pigs weighing from 250 to 500 gm. were obtained from outside sources.

### *Recording of the Results*

Measurements and colored drawings were made of skin lesions at frequent intervals. The lesions were described in terms of the area of skin affected and also the intensity of the inflammation. The extent of tuberculous involvement found at autopsy was recorded according to the following plan. The inguinal, axillary, cervical, tracheal, mesenteric, and iliac lymph nodes were removed cleanly and weighed, and estimates of their involvement made. The spleen was weighed. The extent of the gross pulmonary, hepatic, and renal tuberculosis was estimated and graded from 1 to 4, grade 1 representing minimal involvement and grade 4 maximum involvement. Histological sections were made as a routine procedure.

*The Effect of Testicle Extract on Experimental Tuberculosis in Rabbits*

The effect of dispersion on the lesions induced by intradermal infection was studied with several strains of tubercle bacilli available, which differed markedly in their virulence for rabbits.

*Infection with Bovine Tubercle Bacilli, Strain B-1.*—3 rabbits were injected in the skin of the flank with 1.0 mg. of B-1 cultures suspended in 2.0 cc. of testicle extract, and 3 additional rabbits were similarly injected using saline solution instead of testicle extract.

4 to 6 days after the inoculation the animals injected with the saline suspension developed small nodules at the site of inoculation, and by 16 days these had increased slightly in size and ulceration and drainage of purulent material occurred. These lesions persisted during the 7 weeks of observation, although becoming slightly smaller and less productive after the 4th week.

The 3 animals injected with the testicle extract suspension showed a marked inflammatory reaction in the skin 4 days after inoculation, about 10 times as large as in the controls. During the next 8 to 10 days the inflammatory reaction increased in severity, the skin became indurated, and the central portions underwent necrosis. Following this the entire lesion became ulcerated. Seropurulent material drained from these ulcers for approximately 10 days, after which they became covered with apparently healthy granulation tissue. During the next 4 weeks of observation these ulcers diminished considerably in size. The skin forming the border of these ulcers was not normal healthy skin, however, as in many places small pockets of caseous material had formed. Such lesions are illustrated in Figs. 1, 2, and 3.

The condition of these animals during the 7 weeks of observation was in marked contrast to the 3 control animals injected with the saline suspension. They lost an average of 540 gm. in weight, whereas 2 of the controls maintained their original weight and one registered a slight gain.

50 days after infection the 6 animals were sacrificed. The 3 control animals showed involvement of the regional lymph glands, but no visceral lesions. On the other hand, all of the animals injected with the testicle extract suspension had developed pulmonary tuberculosis, in one instance associated with splenic tuberculosis, and in another with tuberculosis of the testicles.

*Infection with Bovine Type Bacilli, Strain Ravenel.*—2 rabbits were injected in the skin of the flank with 1.0 mg. of Ravenel culture suspended in 2.0 cc. of testicle extract. 2 additional control rabbits were similarly injected with 1.0 mg. of bacilli suspended in 2.0 cc. of physiological salt solution.

The resulting lesions were similar in nature and in their rate of development to the lesions which followed inoculation with the B-1 strain. The animals lost weight during the 50 days of observation, although the 2 injected with testicle extract lost considerably more than the 2 controls. At the end of this time they were sacrificed. The 2 control animals showed a few scattered tubercles in the lungs and kidneys. The 2 animals that had been injected with testicle extract showed widespread pulmonary tuberculosis, extensive renal tuberculosis, and tubercles in the spleen and bone marrow.

*Infection with the Human Strain H 37.*—6 rabbits were injected in the skin of the flank with 1.0 mg. of H 37 bacilli suspended in 2.0 cc. of testicle extract. 3 control rabbits were similarly injected with 1.0 mg. of H 37 bacilli suspended in 2.0 cc. of saline solution.

The skin lesions which developed in these animals were similar to those following inoculation with the 2 bovine strains. However, they developed more quickly and also reached their maximum size earlier. After 7 weeks of observation the entire group was sacrificed. It was found that in none of the animals was there any evidence of visceral infection, although extension of the infection to the local or regional lymph glands had occurred in all.

*Infection with Avian Type Bacilli, Strain Avian 1.*—3 rabbits were injected in the skin of the flank with 2.0 mg. of Avian 1 bacilli suspended in testicle extract, and 3 control rabbits were injected with a similar dose suspended in saline solution.

The lesions resulting from the injection of the testicle extract suspension were markedly increased in size and severity as in the preceding experiments with the human and bovine strains. The increased dose (2.0 mg. instead of 1.0 mg. as in the three preceding experiments) resulted in a more severe local lesion, with ulceration extending into the subcutaneous tissues. The lesions developed more rapidly than those following inoculation with either the human or the bovine

strain. However, when the 6 animals were sacrificed at the end of 4 weeks, no visceral tuberculosis was found in any of them.

*Infection with B.C.G.*—2 rabbits were injected in the skin of the flank with a suspension of 1.0 mg. of B.C.G. in 2.0 cc. of testicle extract, and 2 control rabbits were similarly injected with a suspension in saline solution.

In the animals injected with the suspension in testicle extract there was a marked enhancement of the local lesion, and involvement of the regional lymph nodes, but in no case was there any evidence of extension to the lungs or other viscera.

The foregoing experiments showed that the intradermal injection of relatively large amounts of tubercle bacilli suspended in testicle extract resulted in a marked enhancement of the local lesion. This finding is in agreement with observations made on other bacteria and viruses.

Enhancement of the local lesion was followed by an increase in the visceral lesions, in the case of the two virulent bovine strains. The other strains employed were not capable of producing generalized lesions when the amount used was injected into the skin of rabbits without testicle extract. The addition of this agent did not enable such visceral infection to take place.

*The Dermal Lesions Produced in Rabbits by the Simultaneous Injection of Testicle Extract and Small Quantities of Tubercle Bacilli*

*Experiments with Living Bacilli.*—It has been shown with many bacterial strains that when the number of bacteria injected falls below a certain critical concentration, the action of testicle extract results in the suppression of the lesion rather than an enhancement of it. Experiments were devised to study the possibility of such suppression of lesions in the case of tubercle bacilli.

Suspensions of 5 acid-fast strains were prepared as usual. Each strain was tested by intradermal injection in several dilutions with testicle extract on one flank of a rabbit, and on the other side the same dilutions made with normal saline solution were tested. Each test was repeated on 2 or more animals. The strains used, the quantity of bacteria injected, and the average size and intensity of the lesions resulting in a total of 15 rabbits are given in Table I.

It will be seen from Table I that the spreading action of testicle extract enhances the lesions in the skin in practically all of the dilutions tested, even in those which approach the minimal infective dose, and that this occurred in all of the strains studied. The severity of the lesions produced in the skin by the various strains bears no relation to their ability to invade the tissues of the host.

In two additional tests dealing with the H 37 strain of human tubercle bacilli, and with the Avian 1 strain, each of the dilutions was tested in a single rabbit, thus eliminating the possibility that the production of several lesions in one rabbit would result in interference or anomalous results. The results obtained from 12 rabbits so tested showed again that spreading caused enhancement of the lesions at all dilutions of the bacteria.

As pointed out in Table I pustules resembling those produced by certain filterable viruses and by certain bacteria were observed. In section these proved to be small tubercles, about 2 mm. in diameter. They appeared when small quantities of tubercle bacilli were spread through the skin. As shown in the next section small quantities of dead bacteria, when spread, also produce such lesions.

*Experiments with Heat-Killed and Defatted Bacilli.*—The preceding tests with various quantities of bacilli were repeated using suspensions of bacilli which had been killed by heating in a water bath at 70°C. for 1 hour, and with bovine tubercle bacilli which had been subjected to extraction with an alcohol-ether mixture for 2 months. As in the previous tests each strain was injected with and without testicle extract in the same rabbit, each test save one being repeated 2 or 3 times. Results based on 14 rabbits are given in Table II.

It is clear from Table II that the lesions resulting from the injection of heat-killed bovine (Ravenel), Human H 37, and B.C.G. were considerably smaller than the lesions produced by like amounts of living bacilli, while on the other hand the bovine (B-1) strain and the Avian 1 strain were little affected by heating so far as their ability to produce skin lesions was concerned. In all cases, save the Human H 37 and the Ravenel strain, there was a definite enhancement as a result of spreading with testicle extract. In the case of the 2 strains excepted, the lesions induced were so small that enhancement was doubtful or

TABLE I

*Average Size of Lesions Produced by Spreading Decreasing Quantities of Tubercle Bacilli in the Skin of Normal Rabbits*  
(Recorded at the Time of Maximum Lesion)

| Strain injected       | Amount of culture injected |                  |          |                   |                  |                  |                  |                  |             |                  |              |                  | Time of earliest clear lesion | Time of maximum lesion | No. of tests |
|-----------------------|----------------------------|------------------|----------|-------------------|------------------|------------------|------------------|------------------|-------------|------------------|--------------|------------------|-------------------------------|------------------------|--------------|
|                       | 0.5 mg.                    |                  | 0.05 mg. |                   | 0.005 mg.        |                  | 0.0005 mg.       |                  | 0.00005 mg. |                  | 0.000005 mg. |                  |                               |                        |              |
|                       | Saline                     | Testicle extract | Saline   | Testicle extract  | Saline           | Testicle extract | Saline           | Testicle extract | Saline      | Testicle extract | Saline       | Testicle extract |                               |                        |              |
|                       |                            |                  |          |                   |                  |                  |                  |                  |             |                  |              |                  |                               |                        |              |
| sq. cm.               | sq. cm.                    | sq. cm.          | sq. cm.  | sq. cm.           | sq. cm.          | sq. cm.          | sq. cm.          | sq. cm.          | sq. cm.     | sq. cm.          | sq. cm.      | days             | days                          |                        |              |
| Bovine (B-1).....     | 3.6                        | 29.7             | 0.4      | 9.1               | 0.4              | 18.8             |                  |                  |             |                  |              |                  | 2-5                           | 28-30                  | 3            |
| Bovine (Ravenel)..... | 2.1                        | 16.5             | 0.4      | 21.3 <sub>p</sub> | 0.3              | 1.5              |                  |                  |             |                  |              |                  | 10                            | 30-32                  | 2            |
| Human (H 37)*.....    | 5.0                        | 18.9             | 2.9      | 20.0              | 0.9 <sub>e</sub> | 1.9 <sub>e</sub> | 0.9 <sub>e</sub> | 0.3 <sub>e</sub> | 0.3         | 0.1              | 0.1          | 0.1              | 10                            | 16-20                  | 5            |
| Avian S.....          | 5.0                        | 25.1             | 2.5      | 17.6              | 2.0              | 7.2              | 0.0              | 0.1              | 0.0         | 0.0              | 0.0          | 0.0              | 2-5                           | 15                     | 3            |
| B. C. G.....          | 4.8                        | 17.0             | 2.2      | 9.2               | 1.8              | 3.2 <sub>p</sub> | 0.9              | 0.8 <sub>p</sub> | 0.5         | 0.4              | 0.0          | 0.0              | 2-5                           | 15                     | 2            |

*p* = pustular lesion.

*e* = erythematous lesion.

\* The last three dilutions of the strain were each tested in a single rabbit.

TABLE II

*Average Size of Lesions Produced by Spreading Decreasing Quantities of Heated and Defatted Tubercle Bacilli in Normal Rabbits*  
(Recorded at the Time of the Maximum Lesion)

| Strain injected         | Amount of culture injected |                  |          |                  |           |                  |            |                  |             |                  | Time of earliest clear lesion | Time of maximum lesion | No. of tests |
|-------------------------|----------------------------|------------------|----------|------------------|-----------|------------------|------------|------------------|-------------|------------------|-------------------------------|------------------------|--------------|
|                         | 0.5 mg.                    |                  | 0.05 mg. |                  | 0.005 mg. |                  | 0.0005 mg. |                  | 0.00005 mg. |                  |                               |                        |              |
|                         | Saline                     | Testicle extract | Saline   | Testicle extract | Saline    | Testicle extract | Saline     | Testicle extract | Saline      | Testicle extract |                               |                        |              |
|                         |                            |                  |          |                  |           |                  |            |                  |             |                  |                               |                        |              |
|                         | sq. cm.                    | sq. cm.          | sq. cm.  | sq. cm.          | sq. cm.   | sq. cm.          | sq. cm.    | sq. cm.          | sq. cm.     | sq. cm.          | days                          | days                   |              |
| Heated Bovine B-1.....  | 3.5                        | 24.9             | 1.8      | 5.0              | 0.8       | 1.3              | 0.2        | 0.1              | 0.0         | 0.0              | 4-9                           | 28-30                  | 3            |
| Heated Bovine Ravenel.. |                            | 2.0              | ±        | ±                | ±         | ±                | 0.0        | 0.0              | 0.0         | 0.0              |                               | 30                     | 1            |
| Heated Human H 37....   | 1.0                        | 1.0              | 0.5      | 0.5 <sub>p</sub> | ±         | ±                | 0.0        | 0.0              | 0.0         | 0.0              |                               | 16-20                  | 3            |
| Heated Avian S.....     | 8.2                        | 22.1             | 1.3      | 15.8             | 0.4       | 11.2             | 0.4        | 9.3              | 0.2         | 18.8             | 2-5                           | 15                     | 3            |
| Heated B. C. G.....     | 1.8                        | 4.2              | 1.0      | 1.5              | 0.8       | 0.8              | 0.3        | 0.5              | 0.0         | 0.1              | 2-5                           | 15                     | 2            |
| Defatted Bovine B-1.... | 2.5                        | 5.3              | 1.7      | 0.7              | 0.4       | 0.3              | 0.4        | 0.3              | 0.3         | 0.0              | 2                             | 30                     | 2            |

difficult to estimate.<sup>1</sup> It is also clear that the process of defatting to which the bovine bacilli were subjected rendered them much less capable of inducing severe lesions than either killed or living bacilli. When large quantities of defatted bacilli were spread, enhanced lesions resulted, but when smaller quantities were injected with testicle extract, partial suppression of the lesions occurred.

It would appear from this group of experiments that the tubercle bacilli behave somewhat like the filterable viruses and also like certain virulent bacteria such as *Pneumococcus* Type I, in that when they are injected together with testicle extract, it is not possible to demonstrate any suppression of lesions even when the smallest quantities are injected. This property is retained after exposure to 70°C. for 1 hour. In the case of defatted bacilli, the removal of the lipoids apparently made possible the suppression of lesions after the injection of small quantities with testicle extract. In this sense the defatted bacilli behaved like the non-acid fast bacteria.<sup>2</sup>

#### *The Effect of Testicle Extract on Experimental Tuberculosis in Guinea Pigs*

Some of the foregoing experiments were repeated using guinea pigs instead of rabbits.

6 guinea pigs were injected intradermally in the flank with a suspension of 0.1 mg. of human tubercle bacilli (H 37) in 2.0 cc. of testicle extract. 5 other guinea pigs were similarly injected, using saline solution instead of testicle extract.

5 guinea pigs were injected with a similar amount of bacilli suspended in testicle extract, subcutaneously in the right groin, and 9 controls were injected with the same dose suspended in saline solution.

<sup>1</sup> The explanation of why certain strains of acid-fast bacilli lose more of their ability to produce lesions after being killed by heat than do others awaits further study, which is now being carried on.

<sup>2</sup> As a further illustration of the importance of lipoids in the genesis of lesions after the bacteria have been dispersed, the following results of some experiments with *M. phlei* seem worthy of record. This acid-fast bacillus is completely avirulent for laboratory animals, and induces only very mild lesions after injection into the skin of rabbits. Such lesions, had they been produced by an avirulent non-acid-fast strain, would have been most easily suppressed by the spreading action of testicle extract. However, following the injection of several dilutions of the strain *M. phlei*, both living and after heating at 70°C. for 1 hour, there was a uniform enhancement of all lesions as the result of spreading.



In both groups of guinea pigs the addition of testicle extract caused a marked enhancement of the primary lesion; those inoculated in the skin of the flank developed large shallow ulcers, about 30 to 35 sq. cm. These lesions are illustrated in Figs. 4, 5, and 6. In those animals inoculated in the groin, extensive lesions occurred which involved the entire hypogastrium and extended down the right thigh. Marked induration of the tissues occurred, with splinting of the entire hind quarter. Ulceration and drainage of purulent material occurred at several scattered points. The control animals, injected with saline solution suspension developed typical small nodules at the point of

TABLE III

*The Extent of the Generalized Lesions Found in Guinea Pigs Inoculated with Tubercle Bacilli Suspended in Testicle Extract and Saline Solution Respectively*

|                                  |                  | No. of animals | Average weight |             | Average involvement |       | Time of autopsy |
|----------------------------------|------------------|----------------|----------------|-------------|---------------------|-------|-----------------|
|                                  |                  |                | Spleen         | Lymph nodes | Lungs               | Liver |                 |
|                                  |                  |                | gm.            | gm.         |                     |       | days            |
| Group I, intradermal injection   | Testicle extract | 6              | 6.6            | 7.2         | +++±                | +++   | 48              |
|                                  | Saline solution  | 5              | 3.8            | 7.7         | +++                 | +++   |                 |
| Group II, subcutaneous injection | Testicle extract | 5              | 2.5            |             | +++                 | +++   | 60              |
|                                  | Saline solution  | 9              | 1.5            |             | ++                  | +     |                 |

injection, which ulcerated and drained after the 2nd week of infection.

The animals treated intradermally were sacrificed 48 days after injection and the ones inoculated in the groin were sacrificed after 60 days. The extent of the involvement was recorded and these data are shown in Table III.

It will be seen from Table III that the spreading of the injected bacilli in the skin resulted in a more widespread general disease, especially in the spleen and lungs. This study of the effect of testicle extract on the primary infection of guinea pigs confirms the findings in similar studies on rabbits.

*The Effect of Testicle Extract on the Tuberculin Reaction*

It has been shown (4) that the dispersion of antigens in the skin of a sensitized animal by means of testicle extract results in a marked increase in the size of the ensuing (Arthus or Shwartzman) reaction, which is attended by a corresponding diminution of the severity of the reaction, which may even be completely suppressed. With this in mind, it was decided to study the effects of dispersion of the antigen involved in the production of the tuberculin reaction. At the same time a study was made of the effects of such a modified reaction upon the evolution of the disease.

The following experiments were performed to study the result of adding testicle extract to the tuberculin used in making skin tests in guinea pigs.

5 guinea pigs which had been infected 30 days previously by the subcutaneous injection of 0.1 mg. of human tubercle bacilli (Strain H 37) were inoculated intradermally with mixtures of tuberculo-protein MA 100 (Sharpe and Dohme) and testicle extract. 5 tuberculous guinea pigs were injected with mixtures of tuberculo-protein and salt solution as a control. Each animal was given 1 injection; the amount of tuberculo-protein given was varied from 0.25 mg. to 3.0 mg. This was mixed with 0.7 cc. of testicle extract or 0.7 cc. of saline solution.

At the end of 24 hours the 5 control animals showed strong reactions to the tuberculin, which in the following days became necrotic. In the 5 injected with the mixtures of tuberculo-protein and testicle extract the reactions at the end of 24 hours were diffuse, covering about 20 sq. cm., and showed only a reddening of the skin. These reactions faded rapidly, and at the end of 48 to 72 hours had disappeared with no ensuing necrosis.

A second experiment was performed to determine whether or not the diminution of the intensity of the skin reaction by spreading had any effect upon the essentially toxic action of large doses of tuberculin on tuberculous guinea pigs.

5 tuberculous guinea pigs were injected with 10 mg. of tuberculo-protein, mixed with 1.0 cc. of testicle extract. 5 control tuberculous guinea pigs were injected with 10 mg. of tuberculo-protein mixed with 1.0 cc. of salt solution.

The testicle extract caused dispersion of the tuberculin, as evidenced by the diffuse nature of the reaction, and the reduction of the intensity

of the local reaction per unit area of skin. The symptoms of tuberculin intoxication were in no way reduced by such dispersion; the animals became sick and weak, and several in each group died during the following 14 days. The temperature reaction of both groups of animals to the injections were similar; a rise of 1.5 degrees during the first 6 hours being followed by a return to normal limits.

From these experiments it appears that, although the spreading of tuberculin through the skin of a tuberculous guinea pig results in a reduction in the intensity of the lesion per unit area of tissue, the systemic effects are in no way altered.

### *The Effect of Testicle Extract on the Koch Phenomenon in Rabbits*

The reaction of a tuberculous animal to a second infection is characterized by an acute inflammatory response, with a tendency to localization and destruction of the injected bacilli. The following experiment was performed in order to study the local and general reactions of tuberculous rabbits to the injection of tubercle bacilli dispersed through the skin.

*Experiments with Living Bacilli.*—15 rabbits which had been infected by the intravenous injection of 0.5 mg. of bovine tubercle bacilli Strain B-1, 35 days previously, were divided into 3 groups of 5. One group was injected in the skin with 1.0 mg. of B-1 bacilli suspended in 1.0 cc. of testicle extract; another group was similarly injected with a suspension made in saline solution; and the third group left as controls. 3 previously normal rabbits were injected intradermally with the suspension in testicle extract, and 3 more with the saline suspension, as controls of the viability of the organisms and for comparison of the lesions of primary infection with those of superinfection.

In the tuberculous rabbits there was a rapid response to the injection of bacilli, coming to a maximum within 48 hours. In the animals injected with testicle extract the lesions were very large, averaging 55 sq. cm., in contrast to the animals injected with saline suspensions, which showed small abscesses in the skin averaging about 3 sq. cm. These latter lesions ulcerated and drained purulent material after the 6th to 8th day. After the 2nd week they showed a reduction in size and showed a tendency to heal, which was complete at 6 weeks.

The lesions in the group superinfected with bacilli mixed with testicle extract were large, indurated, raised, and sharply circumscribed.

During the first 2 weeks they became increasingly indurated, although showing less evidence of inflammation; in the subsequent weeks a very slow regression took place, the lesions becoming smaller and less indurated. 6 weeks after injection the lesions were practically healed, with slight scarring of the skin in some instances. Microscopic sections of the skin taken at autopsy showed a layer of epithelioid cells lying just beneath the epithelium. This is illustrated in Fig. 7.

The previously normal animals injected at the same time, with a similar dose of bacilli in testicle extract, developed large ulcerated lesions such as those already described, which drained purulent material for some time and which at the end of 6 weeks were but partially closed.

The entire group of animals were sacrificed 50 days after their injection and it was found that the tuberculous animals which had been superinfected did not show any more visceral lesions than the animals which had received their primary infection at the same time but which were not superinfected. All showed a moderate amount of tuberculosis, limited to the lungs. The 3 previously normal animals which were inoculated with the same suspension with testicle extract showed more advanced pulmonary tuberculosis than in the corresponding animals inoculated with saline suspension of bacilli, and more than either of the groups subjected to superinfection. There was also involvement of the spleen in one case and of the testicles in another case.

The use of testicle extract brought out another striking difference between the reaction of normal and tuberculous rabbits to an inoculation with tubercle bacilli. While the previously normal animals developed large lesions which became ulcerated, the lesions of the tuberculous animals did not undergo ulceration, but simply regressed and healed after reaching a maximum size and intensity.

*Experiments with Dead Bacilli.*—15 rabbits, infected by the intravenous injection of 0.1 mg. of tubercle bacilli (Ravenel) were divided into 3 groups of 5. 47 days after inoculation one group was injected intradermally with 0.1 mg. of killed Ravenel culture, suspended in 2.0 cc. of testicle extract. Another group was similarly injected with killed bacilli suspended in saline solution, and the third group was left as a control. 8 days later the injections were repeated.

As in the foregoing experiment with living bacilli there was a rapid response to the injection, and as usual, the lesions produced by the injection of bacilli with testicle extract were several times larger than those in the controls.

The rapidly progressive disease caused by infection with the Ravenel strain manifested itself in the death of 7 out of 15 animals with far advanced tuberculosis within the next 40 days—a total of 95 days after the original infection. At this date the remainder of the animals was sacrificed. Of the 7 animals that died before this time, 4 were in the group reinjected with bacilli in saline solution, 2 were in the untreated control group, and only 1 in the group injected with the bacilli suspended in testicle extract.

In Table IV the autopsy findings are expressed as an average for the 3 groups.

It is evident from Table IV that the rabbits injected with the bacilli suspended in testicle extract showed less tuberculosis than the group injected with the saline suspension, and also less than the control group. In summarizing, it is evident that the addition of testicle extract to a suspension of tubercle bacilli, living or dead, injected into a tuberculous rabbit, resulted in a marked increase in the size of the local lesion, but without any corresponding diminution of the severity, as was the case in the Arthus, Shwartzman, and tuberculin reactions. The tuberculous rabbits, however, were not only able to resist further infection of the viscera following such injections, but in the case of the last experiment showed a considerable reduction in the extent of the visceral lesions.<sup>3</sup>

#### *The Effect of Spreading Progressively Smaller Quantities of Tubercle Bacilli in the Skin of Tuberculous and Immunized Rabbits*

The same procedure of spreading progressively smaller quantities of bacilli described in the experiments with normal rabbits was employed in tuberculous rabbits and rabbits that had been immunized by repeated injections of heat-killed tubercle bacilli.

The animals were prepared as follows: A group of 5 rabbits were given a total of 12 injections of heat-killed Avian 1 bacilli, over a period of 3 months. 1.0 mg.

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<sup>3</sup> Some additional experiments have shown that intradermal injection of 0.5 mg. of heat-killed Avian 1 bacilli with and without testicle extract into a rabbit prepared by successive injections of heat-killed bacilli of the same strain, resulted in lesions which had the same character as far as size, severity, and evolution were concerned, as those which were produced by the living bacilli.

was given at each dose. The injections were given both intravenously and intradermally, alternately.

Another group of 2 rabbits had been inoculated intravenously with 1.0 mg. of Bovine tubercle bacilli (B-1) 2 months previously. Both groups of animals were injected with progressive dilutions of the corresponding strain of living tubercle bacilli, mixed with testicle extract. Similar injections were made on the opposite flanks, using suspensions in saline solution. The results are expressed in Table V.

It is clear from Table V that spreading enhances the skin lesions resulting from such injections, at all dilutions, in tuberculous rabbits as well as in the immunized ones.

#### *The Effect of Testicle Extract on the Koch Phenomenon in Guinea Pigs*

When tuberculous guinea pigs are reinoculated intracutaneously with tubercle bacilli, the response of the animals to such an injection is characterized by a rapidly developing acute inflammatory reaction. Extension of the infection to the regional lymph glands is prevented or delayed (11), and healing of the local lesion occurs. This state of resistance acquired as a result of tuberculous infection is not complete; as Kraus and others have shown, using animals infected with a strain of tubercle bacilli incapable of producing progressive disease in guinea pigs. Such animals when superinfected with a virulent strain, eventually develop progressive tuberculosis, although far outliving previously normal animals injected with a similar dose.

In the foregoing section we have shown that the addition of testicle extract to the superinfecting dose of bacilli in rabbits does not increase the extent of the visceral lesions, as compared with animals similarly superinfected with bacilli suspended in saline solution. These experiments were repeated on a larger scale using tuberculous guinea pigs to study the effects of such superinfection on their resistance to the disease. A total of 93 guinea pigs were used in five consecutive experiments. The following is a typical protocol.

A group of 30 guinea pigs were inoculated with 0.01 mg. of living human tubercle bacilli, Strain H 37. The injection was made in the right groin. 6 weeks later 10 of these animals were inoculated with a suspension of 0.1 mg. of the same Strain H 37, in testicle extract, the injection being made into the skin of the flank. 10 additional animals of the original 30 were reinoculated with a like dose of bacilli, suspended in saline solution, the remaining 10 being left as controls of the primary infection. The resulting lesions were observed daily, and drawings and measurements of them were made. 6 weeks after superinfection the entire group was sacrificed and autopsies performed.

The reaction in the skin following the superinfection with organisms suspended in testicle extract was greatly enhanced. During the first 48 hours there was an acute inflammation of the skin, with hemorrhage and purple discoloration. The area involved was much greater than in the controls, approximately 15 times, but the intensity of the signs of inflammation during the first 48 hours was about the same.

The controls (superinfected with organisms suspended in saline) developed small nodules in the skin which, after the 4th day, broke down and drained purulent material. At the end of 2 weeks these started to show healing. By this time the lesions resulting from the superinfection with testicle extract had undergone necrosis, with extensive sloughing of the affected skin. The resulting ulcers healed only after many weeks.

Sections taken from such lesions 10 days after superinfection showed that the dispersion of the injected bacilli through the dermis had resulted in the formation of a thin layer of epithelioid cells lying in the collagenous portion of the skin. There was very little caseation, and none of the cellular reaction extended beneath the panniculus carnosus muscle. Sections stained with carbolfuchsin showed the presence of very few tubercle bacilli, in contrast to the great number found in the sections from the control animals. The lesions in the controls were small abscesses, extending into the subcutaneous connective tissue, with a definite limiting zone of connective tissue around them. They were filled with caseous debris.

When the extent of the tuberculosis in the 2 groups was compared, it was found that the animals superinfected with the addition of testicle extract showed less tuberculosis than the animals superinfected with a saline suspension. The spleens were smaller, the extent of hepatic and pulmonary tuberculosis was diminished, and lymphatic involvement was not so marked. These results and those of the additional experiments are given in Table VI.

From Table VI it is seen that there was no apparent difference between the control group and the group superinfected with the saline suspension. Other workers (12) have stated that superinfection, under the proper conditions, has the effect of stimulating the resistance of the animals to the disease. It would seem evident that the conditions of time and dosage in our animals did not allow this effect

**TABLE IV**  
*The Extent of the Lesions in Rabbits Injected with Heat-Killed Tubercle Bacilli  
 Suspended in Testicle Extract and Saline Solution Respectively 47 and 55  
 Days after Infection*

|  | No. of animals | Average weight |             | Average involvement |        |             |
|--|----------------|----------------|-------------|---------------------|--------|-------------|
|  |                | Spleen         | Lymph nodes | Lungs               | Kidney | Bone marrow |
|  |                | gm.            | gm.         |                     |        |             |
| Injected with bacilli suspended in testicle extract..... | 5              | 2.7            | 1.5         | +++                 | ++±    | +±          |
| Injected with bacilli suspended in saline solution.....  | 5              | 2.9            | 2.0         | ++++                | ++++   | ++++        |
| Untreated controls of primary infection.....             | 5              | 2.9            | 3.0         | ++++                | ++++   | ++±         |

**TABLE V**  
*Average Size of Lesions Produced by Spreading Decreasing Quantities of Tubercle  
 Bacilli in the Skin of Immunized Rabbits  
 (Recorded 15 Days after Infection)*

| Strain injected  | Amount of culture injected |                  |          |                  |           |                  |            |                  |             |                  |     |     | No. of tests          | Method of preparation of the animals |
|------------------|----------------------------|------------------|----------|------------------|-----------|------------------|------------|------------------|-------------|------------------|-----|-----|-----------------------|--------------------------------------|
|                  | 0.5 mg.                    |                  | 0.05 mg. |                  | 0.005 mg. |                  | 0.0005 mg. |                  | 0.00005 mg. |                  |     |     |                       |                                      |
|                  | Saline                     | Testicle extract | Saline   | Testicle extract | Saline    | Testicle extract | Saline     | Testicle extract | Saline      | Testicle extract |     |     |                       |                                      |
|                  | sq. cm.                    | sq. cm.          | sq. cm.  | sq. cm.          | sq. cm.   | sq. cm.          | sq. cm.    | sq. cm.          | sq. cm.     | sq. cm.          |     |     |                       |                                      |
| Avian S. ....    | 7.6                        | 20.4             | 4.5      | 14.0             | 1.5       | 14.0             | 0.9        | 1.0              | 0.5         | 1.0              | 0.3 | 5   | Vaccination Infection |                                      |
| Bovine B-1. .... | 2.3                        | 14.1             | 4.6      | 5.0              | 1.2       | 1                | 2          | 0.2              | 0.3         | 0.1              | 0.3 | 0.1 |                       | 2                                    |

TABLE VI

The Extent of Lesions Exposed to Rabbits 15 Days after Infection

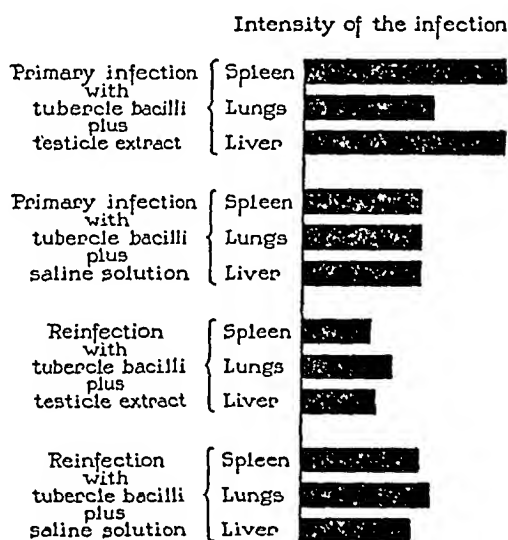
**TABLE VI**  
*The Extent of Lesions Found in Guinea Pigs after Superinfection with Tubercle  
 Bacilli Injected with Testicle Extract and Saline Solution Respectively  
 (Recorded 6 Weeks after Superinfection and 12 Weeks after  
 Primary Infection)*

|                                    | No. of animals | Average weight |             | Average involvement |       |
|------------------------------------|----------------|----------------|-------------|---------------------|-------|
|                                    |                | Spleen         | Lymph nodes | Lungs               | Liver |
|                                    |                | gm.            | gm.         |                     |       |
| Testicle extract.....              | 31             | 3.5            | 5.4         | ++±                 | +±    |
| Saline solution.....               | 36             | 6.0            | 7.4*        | +++                 | +++   |
| Controls of primary infection..... | 26             | 6.1            | 6.2†        | +++                 | +++   |

\* Only 23 animals recorded.  
 † Only 17 animals recorded.



to be demonstrated, as long as the superinfection was made in the usual manner; but that the dispersion of the bacilli did in some fashion swing the balance in favor of the animals' resistance. It would derive perhaps that the rapid and acute inflammatory reaction tended to isolate and discharge the injected bacilli, so that actually very little absorption of bacillary material took place, and that dispersion of the bacilli, by increasing the intimacy with which they came into contact with the tissues and body fluids, increased the absorption of bacillary products. This property of a dispersing agent, of amplifying and accentuating reactions already determined qualitatively,



TEXT-FIG. 1

is especially brought out in the study of tuberculosis, in which a number of complex reactions may occur, one perhaps masking the others.

Text-fig. 1 gives a graphic representation of the differences in the extent of the disease which result from the injection of the tubercle bacilli with and without testicle extract in both normal and tuberculous guinea pigs.

### *The Effect of Parenteral Injections of Testicle Extract on Tuberculous Guinea Pigs*

From the preceding experiments it is seen that the injection of testicle extract together with tubercle bacilli in the normal or tuber-

culous animal results in an enhancement of the local reaction to the injected bacilli. In the previously normal animal this is followed by an increase in the rate of development of visceral lesions, while in the tuberculous guinea pig, the effect is just the opposite. It was decided to test the effect of injecting large amounts of testicle extract parenterally into guinea pigs recently rendered tuberculous.

10 guinea pigs which had been infected with 0.10 mg. of human type H 37 bacilli 10 days prior, were divided into 2 groups of 5 each, one of which was given a series of injections of testicle extract subcutaneously. 5 doses were given during the following 19 days, starting with 2.0 cc. and increasing the dose up to 5.0 cc. A total of 19 cc. was given each animal. 6 days after the last injection the animals were sacrificed for autopsy. The extent of the tuberculosis in the 2 groups is plotted in Table VII.

It is seen from Table VII that there was no striking difference between the 2 groups, and no evidence was obtained that the administration of the testicle extract had either increased the rate of progress of the disease or changed the distribution of the lesions. On the contrary, what slight difference existed was in favor of the treated animals. The introduction of large amounts of the spreading agent into the tuberculous animals had no untoward effect, even in an infection of recent origin (10 days).<sup>4</sup>

*The Effect of Spreading of the Antigen upon Resistance Produced by  
Preventive Vaccination of Guinea Pigs and Rabbits against  
Tuberculosis*

The foregoing experiments, showing the favorable influence that dispersion of the reinfecting bacilli had on the course of the disease, led us to a study devised to increase the immunity conferred upon animals to tuberculosis by repeated injections of heat-killed vaccines.

<sup>4</sup> As a complement to these experiments the following observations are worthy of record. In 3 rabbits with extensive tuberculous lesions in the skin, injections of testicle extract were made into the center and around the edges of the lesions. In no case was there any spread of the infection, and in 2 cases it seemed that a slight acceleration of healing took place.

Henper (13) has reported that similar treatment of lesions induced by *Bacterium leprosum* resulted in a new spread of the infection.

*Experiments on Guinea Pigs.*—A total of 57 guinea pigs were used. 20 guinea pigs were vaccinated with a suspension of heat-killed human tubercle bacilli (H 37) mixed with testicle extract. 19 guinea pigs were vaccinated with a similar suspension in saline solution. A total of 5 injections were given at weekly intervals, into the skin of the flank. In the case of the testicle extract suspensions, the total amount of vaccine was not injected into one area, but divided into 2 portions, one of which was injected into the skin on each side. The first dose was 0.1 mg., the second was 0.2 mg., and the next 3 were 2.0 mg. each suspended in 2.0 cc. of testicle extract and 2.0 cc. of saline solution respectively.

18 days after the last injection of vaccine both groups were given a test inoculation of 0.1 mg. of living human tubercle bacilli (H 37) injected subcutaneously in the right groin. At the same time 18 previously normal guinea pigs were infected with the same amount of bacilli.

50 days after the test infection the entire group of animals was sacrificed and the extent of their visceral lesions compared. The results are recorded in Table VIII.

From Table VIII it will be seen that the extent of the disease in each of the vaccinated groups was less than that in the unvaccinated controls. It is also clear that the animals vaccinated with the testicle extract-vaccine mixture showed much less tuberculosis than either of the other 2 groups.

*Experiments on Rabbits.*—14 rabbits were used. 5 rabbits were given 4 injections of 2.0 mg. of heat-killed bovine tubercle bacilli (Ravenel) mixed with 2.0 cc. of testicle extract, at weekly intervals. The vaccine-testicle extract mixture was divided into 2 portions, and injected into both flanks. 5 rabbits were similarly vaccinated with 2.0 mg. of heat-killed Ravenel, suspended in saline solution. These injections were made in one area only.

17 days after the last injection of vaccine the 10 rabbits together with 4 previously normal rabbits were given a test inoculation of 0.1 mg. of living Ravenel bacilli, injected intradermally in the interscapular region.

Observations of these animals during the next few days disclosed an interesting fact, that the local lesions resulting from the intradermal infection in the group that had been vaccinated with the vaccine-testicle extract mixture were definitely larger, more inflamed, and more indurated than those found in the animals vaccinated with the organisms suspended in saline; these latter were in turn larger and more inflammatory than the lesions produced in the previously normal animals.

It was also observed that in the controls, and in the group vaccinated with the saline suspension the axillary lymph nodes increased

TABLE VII  
Effect of Repeated Injections of Testicle Extract on the Visceral Lesions of Tuberculous Guinea Pigs as Compared with Non-Injected Controls (Recorded 35 Days after Infection)

|                                    | No. of animals | Average weight |             | Average involvement |       |
|------------------------------------|----------------|----------------|-------------|---------------------|-------|
|                                    |                | Spleen         | Lymph nodes | Lungs               | Liver |
| Treated with testicle extract..... | 5              | gm.            | gm.         |                     |       |
| Untreated controls.....            | 5              | 2.3            | 4.0         | +                   | ++±   |
|                                    |                | 3.0            | 5.2         | ±±                  | +++±  |

TABLE VIII  
Extent of Involvement Found in Guinea Pigs Injected with Heat-Killed Tubercle Bacilli Plus Testicle Extract and Saline Solution Respectively, Prior to Their Infection with Tuberculosis (Recorded 50 Days after Infection)

|  | No. of animals | Average weight |             | Average involvement |       |
|--|----------------|----------------|-------------|---------------------|-------|
|  |                | Spleen         | Lymph nodes | Lungs               | Liver |
| Vaccine suspended in testicle extract.....   | 20             | gm.            | gm.         |                     |       |
| Vaccine suspended in saline solution.....    | 19             | 3.5            | 4.6         | ±±                  | ±±    |
| Untreated controls of primary infection..... | 18             | 4.43           | 5.7         | ++                  | ++    |
|  |                | 5.1            | 5.0         | +++±                | +++±  |

TABLE IX  
The Extent of Involvement Found in Rabbits Injected with Heat-Killed Bovine Tubercle Bacilli Plus Testicle Extract and Saline Solution Respectively, Prior to Their Infection with Tuberculosis (Recorded 81 Days after Infection)

|                                       | Rabbit No. | Right axillary lymph node | Left axillary lymph node | Lungs | Kidneys | Intestines |
|---------------------------------------|------------|---------------------------|--------------------------|-------|---------|------------|
| Vaccine suspended in testicle extract | 5i         | 0                         | 0                        |       |         |            |
|                                       | 6i         | 0                         | 0                        | ++    |         |            |
|                                       | 7i         | 0                         | 0                        | +     | ++      | 0          |
|                                       | 8i         | 0                         | 0                        | +     | 0       | 0          |
|                                       | 9i         | +                         | +                        | 0     | 0       | 0          |
| Vaccine suspended in saline solution  | 10i        | ++++                      | ++++                     | 0     | 0       | 0          |
|                                       | 11i        | 0                         | ++++                     | +     | 0       | 0          |
|                                       | 12i        | ++++                      | ++++                     | 0     | 0       | 0          |
|                                       | 13i        | ++++                      | ++++                     | +     | 0       | 0          |
|                                       | 14i        | ++++                      | ++++                     | ++    | 0       | +          |
| Untreated controls primary infection  | 75i        | 0                         | ++++                     | 0     | 0       | 0          |
|                                       | 76i        | ++++                      | ++++                     | ++    | 0       | 0          |
|                                       | 77i        | ++++                      | ++                       | +++   | 0       | 0          |
|                                       | 78i        | ++++                      | +                        | ++    | 0       | 0          |
|                                       | 79i        | ++++                      | ++++                     | ++    | +       | +          |

in size and after 2 weeks became easily palpable. The animals in the group vaccinated with the testicle extract-vaccine mixture showed no palpable enlargement of the regional lymph nodes during the entire period of observation, save in one animal, which had a very slight enlargement. These observations were confirmed at autopsy, the entire group of animals being sacrificed 81 days after the test infection. The results of the autopsies are plotted in Table IX.

It is seen in Table IX that the extent of the disease in each of the vaccinated groups was less than in the unvaccinated controls. It is also clear that the animals vaccinated with the suspensions in testicle extract had considerably less involvement than either of the other 2 groups.

From the two experiments it is concluded that the dispersion of killed tubercle bacilli through the tissues markedly enhanced the degree of immunity conferred, when compared with animals injected with a like number of bacilli suspended in saline solution.

#### DISCUSSION

It has been shown previously with many strains of bacteria that the response of animal hosts to a given experimental infection is very largely affected by the degree of dispersion of the infectious agent through the tissues at the time of inoculation.

Studies have now been made of the effect of dispersion by means of testicle extract on the response of laboratory animals to experimental infection with several strains of tubercle bacilli. Dermal infections were found most suitable, as the extent of dispersion could be readily measured. It has been shown that the spreading of bacilli injected into the skin results in an enhancement of the local lesion, whether the number of bacilli injected was large or small. Enhancement of the local lesion also occurred irrespective of the strain of tubercle bacilli employed. In the case of bacilli virulent for the animal used, such enhancement of the local lesion was accompanied by an acceleration in the rate at which visceral lesions developed. When strains non-virulent for the animal were injected, no extension to the viscera occurred, despite the development of a large local lesion.

The findings emphasize the fact that owing to their peculiar chemical constitution the tubercle bacilli are uniformly capable of producing

the characteristic lesions when introduced into the tissues, entirely irrespective of their ability to invade the tissues of the particular host chosen. It would seem that this accounts for the fact that quantities of bacilli which approach the minimal infective dose are enhanced by dispersion, in contrast to other non-acid-fast bacteria (as for instance the staphylococcus), which may fail entirely to produce a lesion when small quantities are dispersed through the skin. The fact that enhancement occurs similarly with tubercle bacilli killed by heating further emphasizes this point.

In discussing the response of tuberculous animals to superinfection it is necessary to review briefly the reactions to superinfection as such. They are characterized by the rapid localization and destruction of the bacilli, processes which tend to prevent extension of the new process. Under certain conditions, furthermore, superinfection results in a stimulation of the general defense mechanism, resulting in an increased resistance to the already established disease.

Our studies showed that the dispersion of the bacilli employed in superinfection results in an enhancement of the local reaction to the bacilli. The signs of inflammation were more intense, and the lesion was increased greatly in size. In guinea pigs necrosis of the skin occurred, followed by superficial sloughing. The animals in which the bacilli were injected in salt solution developed small abscesses which quickly discharged their contents. Thus both groups of animals demonstrated that at the time of reinfection they were partially immune, as judged by the exhibition of the Koch phenomenon. 6 weeks after superinfection when all were sacrificed and autopsies performed it was found that the animals which had been injected with bacilli mixed with testicle extract showed much less visceral tuberculosis than those receiving the saline suspension of bacilli. It would seem likely that this difference resulted from an increased absorption of bacillary materials, which served as a stimulus to the defense mechanisms of the body. The greatly increased opportunity for such absorption supplied by the dispersion of the bacilli through a large area of skin is obvious. The animals which were superinfected with bacilli suspended in saline solution showed no less tuberculosis than did the untreated controls of primary infection. From this it would seem that under the conditions of our experiments the factors con-

cerned with the localization and elimination of the bacilli in the Koch phenomenon may have acted so efficiently as to preclude the possibility of any good arising from the superinfection, except when the spreading factor was employed.

Studies on the effect of superinfection on the course of tuberculosis in rabbits were not carried out as extensively as in guinea pigs. However, one interesting observation was made which accentuates the difference in the response of these 2 animal species to tuberculous infection. When tuberculous guinea pigs were superinfected in the skin, the appearance and development of the lesions closely simulated a reaction to tuberculin. The inflammatory response was rapid, and went on to necrosis of the skin. There was evidence of existent immunity, since no extension of the second infection to the viscera took place. When tuberculous rabbits were superinfected in the skin, the resulting lesions developed rapidly, but did not resemble in any way a tuberculin reaction; no necrosis or ulceration followed, although a very rapid formation of epithelioid cells did occur. These lesions regressed and healed after reaching a maximum size. This was best shown when the bacilli were dispersed through the skin with testicle extract. Previously normal rabbits developed large lesions which ulcerated and persisted, for many weeks, after a similar infection.

It is well known that rabbits do not exhibit a high degree of skin hypersensitiveness to tuberculin, and yet they do demonstrate a marked resistance to superinfection. Moreover, the elimination of the injected tubercle bacilli by means of abscess formation or by casting off of a slough does not seem to be a necessary element in this resistance to superinfection. It is suggested that the reaction of tuberculous guinea pigs to superinfection is an immune reaction complicated by the presence of allergy, and that the allergy is of aid only as it results in the death and elimination of the infected tissue. In rabbits the reaction is clearly an immune reaction uncomplicated by the presence of allergy.

The vaccination of both rabbits and guinea pigs against tuberculosis with heat-killed cultures of tubercle bacilli was carried out, with and without the use of testicle extract as a dispersing agent. It was shown that the dispersion of the vaccine through the skin resulted in an increased resistance to a test inoculation with living, virulent tubercle bacilli.

The action of testicle extract as a dispersing agent in experimental tuberculosis may be likened to that of an amplifying device. The factors of host immunity and bacterial virulence determine the nature and direction of the response to infection, but the degree of dispersion of the bacilli in the tissues determines to a large extent the magnitude of the response.

#### SUMMARY

1. The skin lesions in rabbits and guinea pigs following intradermal injection of tubercle bacilli (5 strains) were greatly increased in size and severity when testicle extract was added to the inoculum. Such enhancement was followed by a more widespread and rapidly progressing disease only when virulent strains were employed.

2. Attempts to suppress the development of skin lesions resulting from the injection of either normal or tuberculous rabbits with very small quantities of tubercle bacilli mixed with testicle extract were unsuccessful.

3. The skin reactions of tuberculous guinea pigs to tuberculo-protein MA 100 were greatly increased in size and markedly reduced in intensity by the addition of testicle extract to the protein solution. The toxic effect of larger quantities of tuberculo-protein was not altered by the addition of testicle extract.

4. The dispersion of tubercle bacilli through the skin of tuberculous rabbits resulted in a marked enhancement of the Koch phenomenon but was not followed by any extension of the new infection to the viscera. Tuberculous rabbits injected on two occasions with dead tubercle bacilli suspended in testicle extract showed an increased resistance to the disease when compared with controls receiving dead bacilli suspended in saline solution.

5. The resistance conferred upon tuberculous guinea pigs by superinfection was greatly increased when the bacilli employed were dispersed through the skin with testicle extract.

6. The parenteral administration of large quantities of testicle extract to recently infected guinea pigs did not result in any increase in the extent of the visceral lesions.

7. The partial immunity conferred upon guinea pigs and rabbits by vaccination with heat-killed tubercle bacilli was increased as a result of dispersion of the vaccine through the skin with testicle extract.



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## EXPLANATION OF PLATES

## PLATE 1

FIG. 1. Rabbit 143-H. Primary infection, 16 days after inoculation with 1.0 mg. of bovine (B-1) bacilli, suspended in 2.0 cc. of saline solution.

FIG. 2. Rabbit 2-H. Primary infection, 16 days after inoculation with 1.0 mg. of bovine (B-1) bacilli, suspended in 2.0 cc. of testicle extract.

FIG. 3. Rabbit 52-H. Primary infection, 34 days after inoculation with 1.0 mg. of bovine (B-1) bacilli, suspended in 2.0 cc. of testicle extract.

## PLATE 2

FIG. 4. Guinea Pigs 7-86 (upper) and 7-95 (lower). Primary infection. Guinea Pig 7-86 inoculated 36 days previously with 0.1 mg. of human (H 37) bacilli suspended in 2.0 cc. of testicle extract. Guinea Pig 7-95 was inoculated at the same time with 0.1 mg. suspended in 2.0 cc. saline solution.

FIG. 5. Guinea Pigs 22-H and 25-H, 4 days after superinfection with 0.1 mg. of human (H 37) bacilli. Guinea Pig 25-H (upper) received bacilli suspended in saline solution; Guinea Pig 22-H (lower) received bacilli suspended in testicle extract. Arrows point to the limits of swelling.

FIG. 6. Guinea Pigs 18-H and 14-H, 18 days after superinfection with 0.1 mg. of human (H 37) bacilli. Guinea Pig 18-H (upper) received bacilli suspended in saline solution. Guinea Pig 14-H (lower) received bacilli suspended in testicle extract.

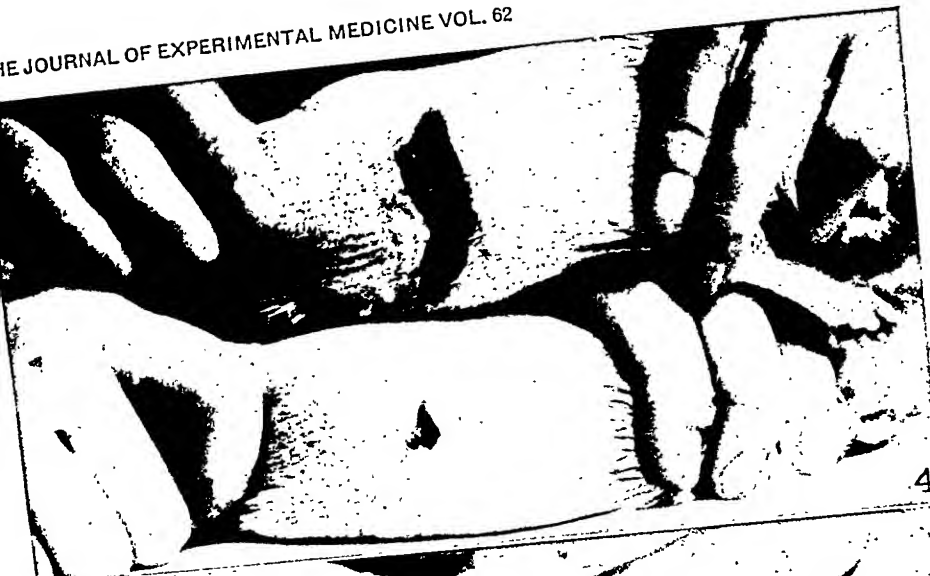
## PLATE 3

FIG. 7. Rabbit 55-H. Section of skin lesion taken 50 days after superinfection with bovine tubercle bacilli suspended in testicle extract. Arrow points to layer of epithelioid cells.

Stained with Masson's trichrome stain. Magnification  $\times 50$ . No color filter used. Light diffused through ground glass.













# IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

I. PREPARATION OF ELEMENTARY BODIES OF VACCINIA  
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## PLATE 4

(Received for publication, March 25, 1935)

In 1906 Paschen (1) observed the elementary bodies of vaccinia in specially stained preparations of *Kinderlymphe*. For many years the importance of this observation was not fully appreciated, because, in addition to the elementary bodies in the preparations, many other small objects which one always had difficulty in distinguishing from the elementary bodies themselves invariably took the stain. In fact, for a long time many investigators either denied the existence of such bodies or doubted their significance. But since Ledingham (2) and Craigie (3) described methods of obtaining elementary bodies of vaccinia in a state of relative purity, a great increase of interest in these minute structures has occurred.

Certain immunological, serological, and chemical investigations of the etiological agent of vaccinia are possible only when one is in possession of considerable amounts of the agent in a state of relative purity. When Craigie described his method of obtaining appreciable quantities of washed elementary bodies of vaccinia, we realized that investigations long delayed because of technical difficulties could be undertaken if his findings were correct. Consequently, we decided to determine whether we could obtain by his method relatively pure elementary bodies in amounts sufficient for our serological and chemical studies. The method is of sufficient importance to warrant another description of it in detail. This is especially true in view of the fact that the validity of results of work to be reported in other papers of the series will depend largely upon the care and accuracy with which preparations of washed elementary bodies are made.



## EXPERIMENTAL

We shall describe (1) the manner in which washed elementary bodies of vaccinia are prepared, (2) the method of hyperimmunizing rabbits in order to obtain potent antivaccinal serum, and (3) the techniques of conducting agglutination tests with elementary bodies and precipitation reactions with virus-free filtrates obtained from emulsions of tissues infected with vaccine virus.

*Virus.*—The vaccine virus used by us was obtained from Craigie and has been designated by him as the C. L. strain. It was secured by him from calf lymph, but for a long time has been passed only through rabbits by means of inoculation on the shaved skin. In order to get a good yield of elementary bodies, a suspension of them must be employed as an inoculum. If one inoculates the skin of a rabbit with testicular vaccine virus, the yield of elementary bodies is small. If, however, the elementary bodies secured in this manner are used as seed for a second passage of the virus in the skin, much larger quantities of bodies are obtained. After several passages of the virus have been made in the skin of rabbits by means of washed elementary bodies large amounts of these structures are regularly secured from each harvest.

*Preparation of Washed Elementary Bodies*

Washed elementary bodies of vaccinia are prepared in the following manner.

Large healthy rabbits with skin free from pigmentation and from excessively coarse hair are selected. The hair is removed from the backs and flanks of the animals by means of a clipping machine and then the skin is carefully shaved and washed. It is scarified and inoculated with the seed virus by means of a small pad of 100 mesh wire gauze held in a surgical clamp; the inoculum is applied to the skin drop by drop during the process of scarification. In this manner an even distribution of virus is procured. Care must be taken to avoid too vigorous scarification of the skin, inasmuch as the exudation of an excessive amount of fluid or blood leads to the formation of crusts over the vaccinal lesions which interfere with the successful preparation of the elementary bodies. Properly prepared and seeded skin will present immediately after inoculation nothing more than a diffuse redness.

On the 3rd day after inoculation, the skin, now considerably thicker than normal, is covered with a confluent vaccinal eruption in which the appearance of vesicles or pustules is rare. At this time the virus is harvested, because it has been found by experience that a 3 day eruption yields a suspension from which the largest amount of relatively pure elementary bodies can be obtained.

In order to harvest the virus, an infected rabbit is sacrificed by the intravenous

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injection of air. Immediately after the death of the animal its skin is quickly removed and pinned to a level flat board. Ether is poured over the skin 2 or 3 times, the excess being removed each time with a cotton pledget. Then 10 cc. of a buffer solution (0.004 M citric acid-disodium phosphate, pH 7.0-7.2) are placed on the skin and spread over the entire infected surface. While the skin is covered with the buffer solution its surface is quickly scraped with a scalpel. The turbid suspension produced in this manner is taken up by means of a spoon and placed in a test tube. The skin is covered with another 10 cc. of buffer solution and scraped a second time. The material secured is mixed with that obtained as a result of the first scraping. The mixture after being shaken vigorously by hand is centrifuged (horizontal centrifuge) for 5 minutes at 3000 R.P.M. The supernatant fluid is decanted and saved. The sediment is then thoroughly mixed with 10 cc. of buffer solution by vigorous shaking and centrifuged again. The supernatant fluid from the second centrifugation is decanted and mixed with the first supernatant material. The sediment is discarded. The pooled supernatant fluids are then centrifuged (horizontal centrifuge) for 5 minutes at 3000 R.P.M. The supernatant fluid is decanted and saved. The sediment is discarded. It is essential that the time which elapses between the death of the rabbit and centrifugation be as brief as possible.

The opalescent fluid obtained in the manner described above is then centrifuged for 1 hour at 4500 R.P.M. in a Bolaget angle centrifuge. Flat tubes, having a length of 11 cm. and internal diameters of 3 and 14 mm. are used. The clear supernatant fluid is decanted and saved, inasmuch as it contains a soluble substance that produces a precipitate when mixed with antivaccinal serum. The sediment is resuspended in buffer solution and centrifuged again. This procedure is repeated 3 times, the supernatant fluid being discarded each time and the sediment resuspended in buffer solution. The sediment from the final centrifugation is taken up in a volume of buffer solution approximately equal to that originally used (20 cc.) and centrifuged in a horizontal centrifuge for 1 hour at 3000 R.P.M. in order to remove bacteria and cellular debris. The supernatant fluid containing the elementary bodies is removed by means of a pipette and stored at  $+4^{\circ}\text{C}$ .

The elementary bodies secured in the manner described represent a stable suspension in a buffer solution. Smears prepared from such a suspension and stained according to Morosow's method (4) contain large numbers of elementary bodies and few or no other formed particles (Fig. 1). In fact, such a preparation reminds one of a smear made from a pure culture composed of extremely small cocci. Furthermore, bacterial counts made by means of poured plate cultures reveal only 200-300 bacteria per cc. Intradermal inoculation of 0.25 cc. of  $10^{-7}$  or  $10^{-8}$  dilutions of the suspensions induces a typical vaccinal lesion.

Suspensions of elementary bodies in buffer solution are stable and suitable for agglutination tests. For chemical analysis, however, the bodies are washed 3 additional times in sterile distilled water. For this procedure the angle centrifuge is used. Distilled water agglutinates elementary bodies, but that phenomenon does not interfere with chemical investigations. The bodies after the final washing are taken up in a small amount of distilled water, frozen, and dried in a desiccator. The resulting product is a white voluminous powder. From each infected rabbit approximately 2 mg. of dried elementary bodies can be obtained. For the preparation of each batch of elementary bodies to be used for chemical analysis at least 10 rabbits are used and in this way we usually secure 20 mg. of material.

*Preparation of the Soluble Flocculable Substance or Substances Derived from Vaccinal Emulsions*

It was stated above that the supernatant fluid resulting from the first sedimentation of elementary bodies in the angle centrifuge contains a soluble flocculable substance or substances. After centrifugation, however, a certain amount of finely divided material and a few elementary bodies remain in the supernatant fluid and must be removed by filtration if accurate immunological observations on the soluble antigens are desired.

In order to obtain a clear fluid for precipitin reactions the supernatant fluid is passed through a Seitz filter pad which has already had passed through it 10 cc. of 10 per cent normal rabbit serum in a buffer solution followed by 10 cc. of the buffer solution alone. The preparation of the pad in this manner is carried out in order to decrease the loss of soluble antigens through adsorption.

The solution resulting from filtration through Seitz pads is perfectly clear and is suitable for use in precipitation tests. Nevertheless, a Seitz filtrate is not always entirely free from elementary bodies. Although these structures may be present in such small numbers as not to interfere with the precipitation reactions, their complete removal is essential when a virus-free filtrate is desired. This is accomplished by means of collodion membranes.

The collodion membranes are prepared by Dr. Bauer according to the method of Elford and have already been described (5). For our investigations membranes

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having an average pore diameter of  $103\ \mu\mu$  were chosen because they invariably hold back vaccine virus which, according to Elford and Andrewes (6), has a diameter of  $125\text{--}175\ \mu\mu$ .

When the supernatant fluid resulting from the first centrifugation of the elementary body suspension in the angle centrifuge is passed through the collodion membranes described above, the soluble antigen is obtained in undiminished titer free from virus as determined by serial testicular passages in rabbits of samples of the filtrate.

#### *Preparation of Potent Antivaccinal Serum*

In order to obtain an antivaccinal serum of high titer rabbits are hyperimmunized in the following manner.

Rabbits convalescent from an intradermal infection with vaccine virus receive 3 intravenous injections of 1.0, 2.0, and 3.0 cc. respectively of washed elementary bodies at weekly intervals. The animals are bled a week after the last injection. Some animals produce better antisera than do others. Consequently, the best sera are chosen for experimental work.

Hyperimmune serum prepared in the manner described will agglutinate washed elementary bodies in dilutions of 1-128 to 1-512 and will produce a precipitate in virus-free filtrates in dilutions of 1-32 or 1-64. Some workers give the rabbits a large number of immunizing doses of the virus and have obtained sera with titers higher than ours. So far we have not found it essential for our work to have sera of extremely high titers, and our method of hyperimmunization does not require a long period of time.

#### *Method of Conducting Agglutination and Precipitation Reactions*

Agglutination and precipitation reactions are carried out according to the method described by Craigie (3).

Pyrex test tubes with outside dimensions of  $10 \times 75$  mm. are used. As a diluent in the tests, physiological saline solution brought to a pH of 7.0 by the addition of 1 cc. of a 0.2 M disodium phosphate-citric acid buffer to each 100 cc. of the solution is employed. The saline solution must be made for each test with recently boiled distilled water. The use of other diluents frequently causes a non-specific agglutination of the elementary bodies. For agglutination a light suspension of elementary bodies yields the best results, and we have found that our stock suspension of bodies diluted four times is satisfactory. The proper dilution of the

filtrates used in precipitation reactions must be determined for each filtrate by preliminary titrations (7). 0.25 cc. amounts of different dilutions of immune serum are placed in a number of tubes. To each tube containing serum an equal amount of diluted elementary body suspension or filtrate is added. Controls in which normal rabbit serum is used always parallel the tests. The tubes with their contents are placed in specially prepared wooden racks and incubated at 50°C. for 18–20 hours. Evaporation of the fluid is prevented by means of a lid, consisting of a strip of wood to which is fixed sponge rubber covered by a thin rubber dam, held in place over the tubes by means of springs (7).

When agglutination and precipitation reactions are carefully conducted in the manner described, consistent and convincing results are obtained. The results are easily read with the unaided eye. A magnifying lens is often of service, however, in determining the exact end-point. Care should be taken not to shake the tubes too violently, because agglutinated elementary bodies are almost completely dispersed by excessive agitation and a precipitate is easily broken up into tiny flocculi difficult to see.

### *Specificity of the Agglutination and Precipitation Reactions*

Some investigators (8) have suggested that the agglutinations and precipitations observed to take place when antivaccinal serum is mixed with emulsions of vaccine virus represent the agglutination of bacteria or the precipitation of bacterial substances in the virus emulsions by antibodies for bacteria in the antiviral serum. Such suggestions were engendered by the fact that much of the original serological work on vaccine virus was conducted with material containing large numbers of bacteria. The work of Burgess, Craigie, and Tulloch (10), Craigie and Tulloch (7), Craigie (3), and others has shown that the reactions under discussion are specific for vaccine virus. Additional evidence of the specificity of the reactions is afforded by the following experiments.

Rabbits that had received an intradermal inoculation of bacteria-free vaccine virus possessed in their sera agglutinins for washed elementary bodies and precipitins that produced a flocculation in virus-free emulsions made from tissue infected with vaccine virus. The titer of these antibodies was increased if the rabbits received repeated injections of bacteria-free virus.

A quantity of bacteria-free testicular vaccine virus, frozen and dried in a vacuum, was extracted with ether in order to remove lipoids which interfere with

the precipitation reaction. 1 gm. of the dried ether-extracted material was added to 100 cc. of physiological saline solution. After extraction had taken place, the material was centrifuged and the supernatant fluid was passed through a prepared Seitz pad. The filtrate prepared in such a manner yielded a precipitate when mixed with the serum of a rabbit that had been immunized against washed elementary bodies but not with normal rabbit serum.

#### DISCUSSION

For a long time (9, 11) it has been known that a precipitate forms when vaccine virus is added to antivaccinal serum. Only recently, however, has it been shown that the precipitate formed under such conditions is due both to the agglutination of elementary bodies and the precipitation of a soluble substance (3). Since Craigie has developed a method of obtaining large amounts of relatively pure elementary bodies and since the flocculable substance can be secured free from the virus, rapid progress should be made in the analysis of these two antigens. Furthermore, the experiments with washed elementary bodies show definitely that these structures are intimately associated with vaccine virus or that they constitute the virus. The exact nature of the bodies still remains to be determined.

#### SUMMARY

By means of differential centrifugation in the horizontal and angle centrifuges it is possible to obtain appreciable quantities of relatively pure elementary bodies of vaccinia. Such preparations of elementary bodies exhibit an extremely high titer of vaccine virus.

The elementary bodies are specifically agglutinated by serum from rabbits immunized by means of injections of bacteria-free vaccine virus.

Virus-free filtrates of dermal vaccine virus and of bacteria-free testicular vaccine virus contain a soluble substance or substances that is precipitated by antivaccinal sera.

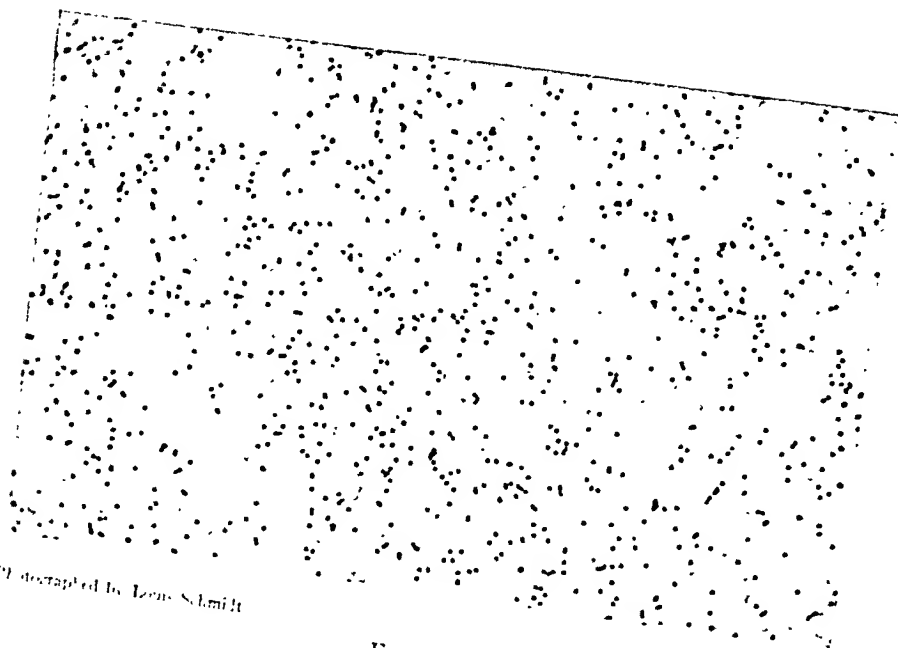
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## EXPLANATION OF PLATE 4

FIG. 1. Washed elementary bodies. Morosow's stain.  $\times 1000$ .



Photographed by Leon Schmitt

FIG. 1





# INTERSTITIAL BRONCHOPNEUMONIA

## I. SIMILARITY OF A TOXIN PNEUMONIA TO THAT PRODUCED BY THE VIRUSES

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PLATES 5 TO 7

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In 1861, Bartels (1) described the presence of a number of cells, many of which were apparently monocytes, in the interstitial tissue of the lungs of patients dying of pneumonia complicating measles. Delafield (2), in 1884, stated that this type of interstitial reaction was quite common in pneumonias. Interest in this type of pneumonia was renewed during the world war by MacCallum (3) and Opie and his associates (4). The cases reported by MacCallum followed measles and those of Opie complicated influenza. MacCallum (3) interpreted the interstitial mononuclear reaction as evidence of a partial immunity of the host to the bacteria causing the pneumonia. He also gave it the name interstitial bronchopneumonia.<sup>1</sup>

Muckenfuss and his associates (5), in 1929, showed that small amounts of vaccine virus injected into the trachea of rabbits caused an interstitial proliferative mononuclear reaction in the lungs, while larger amounts produced an edematous, hemorrhagic consolidation with irregular areas of necrosis. The former type of reaction had been previously noted in psittacosis by Rivers and Berry (6) and others. Later McCordock and Muckenfuss (7) produced lesions similar to those observed in an interstitial bronchopneumonia by following the

<sup>1</sup> Interstitial bronchopneumonia is a thickening of the interstitial tissue of the lungs. This thickening is particularly noted around the bronchi and is due to an increase in the number of mononuclear cells present. In addition to this change in the interstitial tissue there is also involvement of the bronchioles and surrounding alveoli with the usual polymorphonuclear response. Many of the alveoli, however, are filled with either large mononuclear cells or fibrin.

injections of virus with intratracheal inoculations of bacteria. From a study of a number of pneumonic lesions and from reports in the literature, they concluded that interstitial bronchopneumonia occurred after measles, pertussis, and epidemic influenza. Measles is a recognized virus disease. They averred, furthermore, that pertussis and influenza are probably virus diseases on the grounds of a similarity of the anatomical lesions associated with them to those produced by intratracheal injections of vaccine virus. The finding of inclusion bodies in pertussis by McCordock (8) and Rich (9) was given as additional proof. Hence they regarded interstitial bronchopneumonia as the typical response of the lungs to the combined action of a virus and a bacterium, although in no sense specific for a particular virus or bacterium.

While no one doubts that the combination of virus and bacterium will cause such an interstitial reaction, there is some question whether this is the only agent or agents which will produce such a reaction. Olcott (10) reports three cases of acute Friedländer's bacillus pneumonia in which the mononuclear cells predominated. Blake (11) states that extreme care should be used in attempts to relate mononuclear cells to virus or bacterial infections. Foot (12) says that mononuclear cells are much more frequent in pneumonia than is generally thought. In examining the lungs from a number of cases in which there was no known virus infection, we have frequently found sufficient mononuclear and interstitial reaction to make the diagnosis of interstitial bronchopneumonia. Of course it is possible that there was an unrecognized virus infection, but it seems more likely that the reaction was dependent on some bacterial products. As toxins react directly on cells and display certain activities similar to those of viruses (high grade immunity following infection, appearance of specific neutralizing substances in the blood of an infected host, and potency as an antigen), it was thought that a study of the reaction produced in lungs by toxins would aid in understanding the nature of this type of pneumonia. With this idea in mind the following experiments were undertaken.

### *Methods and Materials*

The animals used in the experiments were young adult rabbits. Only rabbits which were not carriers of *Bacterium lepi-septicum* and *Bacillus bronchisepticus*

were used. The presence or absence of these organisms in the nares was determined by means of cultures after silver nitrate instillations, as suggested by Meyer (13).

**Toxins.**—Staphylococcus toxin was made from the strain of *Staphylococcus aureus* employed by Rigdon and his associates (14), and was prepared by the method described by Parker, Hopkins, and Gunther (15).

The streptococcus toxin was prepared from a strain of *Streptococcus hemolyticus* which was isolated from a case of erysipelas. The toxin was made by filtering through a Seitz filter a culture of the organism grown for 7 days in beef infusion broth containing 0.5 per cent blood and 0.5 per cent dextrose.

The diphtheria toxin was prepared by diluting with normal saline the dried toxin obtained from the North Carolina State Laboratory of Hygiene.

**Inoculation.**—The animals were lightly anesthetized with ether. The hair over the neck region was clipped and then iodine poured over the denuded skin. A small incision was made in the midline just below the larynx. The trachea was exposed and the material, which was contained in a tuberculin syringe fitted with a 27 gauge needle, was injected directly into its lumen. The head of the animal was then elevated and the incision was closed with one suture.

**Dose.**—In every instance the total volume of material injected was 1.0 cc. Normal saline was always the diluent except in a few of the earlier experiments where beef extract broth was used. It was found in preliminary experiments that doses of from 0.1 to 0.3 cc. of the staphylococcus toxin produced a proliferative type of lesion, while larger doses produced a hemorrhagic and necrotizing lesion.

**Necropsy.**—Most of the animals were killed 72 hours after injections of the *Staphylococcus aureus* toxin, as the lesions were found at this time to be most actively proliferative. A number of animals injected with the *Staphylococcus aureus* toxin were killed at intervals of from 3 hours to 2 weeks after inoculation. No symptoms were noted in the animals during the progress of the infection except in the case of two half grown rabbits that died shortly after the injections were made. These rabbits were slightly dyspneic.

Animals were killed by a blow at the base of the skull and the necropsies were performed at once. The pleural cavity was opened aseptically, and a portion of the lung at the point of maximum involvement was cut out with sterile scissors. This portion was divided into three parts. One part was streaked on a blood agar plate, and each of the two other pieces was placed in a tube of beef infusion broth. One tube was sealed with vaseline for anaerobic growth. The plate and broth tubes were incubated for 7 days before being reported as sterile.

**Fixation and Stains.**—After the lungs were removed from the body they were sectioned and fixed in Zenker's (Helly modification) solution and 10 per cent formalin. The sections were embedded in paraffin and stained with hematoxylin and eosin and for bacteria according to MacCallum's method. The lungs of 8 rabbits were not treated as described above, but for the sake of better histological preparations were removed without culturing, inflated with air through the bron-

chi, and then fixed *in toto* by injection of Zenker's fluid through the pulmonary artery. After fixation the lungs were sectioned and stained with hematoxylin and eosin and for bacteria as described above.

#### EXPERIMENTAL

*Controls.*—In order to be certain that the reactions to be described in the lungs were due to the toxin used and not to extraneous substances which may have been in the injected material or to a dormant virus, the following control experiments were conducted. In all cases the animals were killed 72 hours after inoculation.

*Heated Toxin.*—3 rabbits were inoculated with 0.3 cc. of the *Staphylococcus aureus* toxin which had been heated to 60°C. for 1 hour. A few focal areas showing slight proliferative changes were found.

*Toxoid.*—2 rabbits were injected intratracheally with 0.3 cc. of a staphylococcus toxoid obtained from the Lederle Laboratories. A few small areas showing a slight increase in mononuclear cells were seen.

*Staphylococcus albus.*—4 rabbits were injected with material prepared exactly as was the *Staphylococcus aureus* toxin except that a *Staphylococcus albus* was used. This material produced slight lesions when 1.0 cc. of the undiluted material was injected. Doses smaller than this resulted only in the accumulation of small numbers of polymorphonuclear cells in the first 48 hours.

*Toxin-Antitoxin Mixtures.*—5 rabbits were injected with a mixture of the *Staphylococcus aureus* toxin and varying amounts of Lederle staphylococcus antitoxin. The mixture was allowed to stand at room temperature for 1 hour before injection. No reaction was observed in the lungs of 4 which received more than 0.1 cc. of antitoxin for 0.4 cc. of the toxin. In one animal which received 0.1 cc. of the antitoxin and 0.9 cc. of toxin a slight proliferation of the lining cells of the alveoli was noticed.

*Actively Immunized Animals.*—2 rabbits were immunized by subcutaneous injections of *Staphylococcus aureus* toxin, starting with 0.1 cc. and increasing the dose until they were receiving injections of 2 cc. 4 weeks after the last immunizing dose they were injected intratracheally with 1 cc. of the toxin. The immunizing injections reduced the amount of reaction in the lungs but did not entirely prevent it. Protection tests conducted in mice showed that 0.05 cc. of the rabbit serum protected mice against a lethal dose of the toxin.

*Passively Immunized Animals.*—6 rabbits received Lederle staphylococcus antitoxin intravenously 24 hours before the intratracheal injections of toxin were made. Doses of serum of from 1 to 8 cc. failed to prevent the development of some mononuclear response, although the reaction was much less than in the non-immunized animals.

*Virus Control.*—A rabbit lung with a typical lesion was triturated with an abrasive. A 10 per cent suspension was made and 1 cc. of this was injected into

each of 3 rabbits. The lungs of these rabbits showed some red blood cells in the alveoli but no mononuclear or proliferative reaction.

*Experiments.*—As it has been shown by Muckenfuss and his associates (5) that small amounts of vaccine virus injected into the lungs of rabbits cause a proliferative and mononuclear reaction, while larger amounts produce an edematous, hemorrhagic consolidation with irregular areas of necrosis, the following experiments were designed to study the effects of both small and large amounts of a toxin. The staphylococcus toxin was chosen for the bulk of the experiments, inasmuch as it was found to produce satisfactory lesions consistently when used in small amounts. The results were checked in later experiments with other kinds of toxins. As the only significant changes were found in the lungs, data concerning the other organs will not be given. Experiment 1 was designed to show the effect of the small amounts of toxin, while Experiment 2 deals with larger amounts of the same agent. As 0.3 cc. of the toxin produced proliferative lesions in some rabbits and necrotizing lesions in others, the rabbits receiving this dose were arbitrarily placed in either Experiment 1 or 2 on the basis of the lesion produced.

*Experiment 1.*—24 rabbits were used in the experiment, and each received either 0.15 or 0.3 cc. of the staphylococcus toxin. 12 rabbits were killed 3, 6, 12, 24, and 48 hours and 7 and 14 days respectively after injection, and 12 rabbits were killed at 72 hours, a time at which the proliferative reaction had been found to be at its height.

*Morbid Anatomy.* *Gross.*—No appreciable change is seen until 12 hours have elapsed since inoculation, at which time the capillaries in part of the lungs are engorged, but little or no edema is present. At the end of 48 hours scattered areas of consolidation are evident. These areas are purple and their surface on section is moist. The lungs at the end of 7 days exhibit firm grey areas which on section are grey, dry, and translucent. After 14 days there is no further change in the appearance of the lungs. Throughout the experiment the pleura remains smooth and glistening.

*Microscopic.*—Animals killed 3 hours after inoculation have a polymorphonuclear cell reaction which is present throughout the interstitial tissue of the lung and is most intense around the bronchioles and blood vessels. There is, however, no phlebitis or arteritis. A few alveoli contain polymorphonuclear cells and an occasional mononuclear cell. There is a slight amount of edema present in the walls of the bronchioles and in the adventitia of the blood vessels. Within 24 hours after the inoculation many alveoli, as well as the interstitial tissue, contain a large number of polymorphonuclear cells. There is also considerable edema.

After 48 hours the number of polymorphonuclear cells and the amount of edema have increased. At this time, however, one sees the first evidence of another reaction; namely, thickening of the alveolar walls. The cells causing the thickening are round or cuboidal with vesicular nuclei and scanty, poorly stained cytoplasm. At this time no attempt will be made to state the nature of these cells or to say whether they have the ability to change into phagocytic cells. They are generally seen lining the alveoli but may also be observed as solid masses or as a syncytium of cells. In addition there are a few cells with many nuclei which resemble foreign body giant cells, and a number of monocytes and macrophages (Fig. 3).

At 72 hours the alveolar walls are markedly thickened, and in places on superficial examination there appears to be a large number of bronchioles (Figs. 1 and 2). On closer inspection these are seen to be alveoli which are lined with large cells having vesicular nuclei, small nucleoli, and scanty, poorly stained cytoplasm. Many of the nuclei are in various stages of mitosis (Figs. 5 and 6). Mixed with these cells are degenerating polymorphonuclear cells, debris, and red blood cells. Extensive hemorrhage is present in other alveoli. The perivascular lymphatics are filled with a large number of lymphocytes and some mononuclear cells (Fig. 4).

At the end of 7 days one sees areas in the lungs in which the lumens of the alveoli are obliterated by the proliferation of cells (Fig. 7). In other areas, however, alveoli containing and lined with large cells are still found. These cells have round or slightly oval vesicular nuclei and scanty cytoplasm and are thought to be proliferating epithelial lining cells. A few mitotic figures are seen. Besides these elements an occasional polymorphonuclear cell and a small number of mononuclear cells with eosin-staining granules in the cytoplasm are found. The granules are about the size of the granules in rabbit polymorphonuclear cells.

The 14 day rabbit lungs resemble in every way the 7 day ones with the exception that fewer cells are seen.

Although no studies were made later than 14 days after inoculation, it is thought, since no fibrous tissue was seen, that the lungs would return to normal if sufficient time were allowed.

*Experiment 2.*—8 rabbits were used, each of which received intratracheally from 0.3 to 0.5 cc. of toxin. In addition to these animals, each of 3 baby rabbits received 0.1 cc. of toxin. Some of the rabbits died within 18 hours, but the majority were killed at the end of 72 hours.

*Morbid Anatomy. Gross.*—All the animals regardless of the time of death have boggy consolidated lungs. The pleural cavities contain slightly more fluid than usual. Numerous purple areas of consolidation are seen. The cut surfaces of the lungs are covered with a large amount of bloody fluid. Some infarct-like areas of necrosis are seen. When the areas extend to the pleura, it is roughened; but in uninvolved areas it is smooth and glistening.

*Microscopic.*—The main difference between lesions observed in this experiment and those described in Experiment 1 is that here, in the beginning, the epithelial

tissue and the underlying structures are actually killed and no proliferation takes place (Fig. 8). These necrotic areas are infiltrated with a large number of polymorphonuclear leukocytes, and healing of them results in the formation of definite fibrous tissue.

In order to ascertain whether the results of the above experiment with staphylococcus toxin could be obtained with other toxins the following experiment was performed.

*Experiment 3.*—11 rabbits were used in this experiment. All of the animals were killed 72 hours after the injection of the toxin. 25, 50, and 100 skin test doses of the diphtheria toxin were given to 5 rabbits and 0.3 and 0.5 cc. of the streptococcus toxin were employed in 6 rabbits.

*Morbid Anatomy.*—The lungs from these rabbits resembled in every detail those described in Experiment 1.

### *Summary of Experiments*

The injection of relatively small amounts of toxin into the trachea of rabbits resulted in a reaction in the tissue evidenced by an exudation of large numbers of polymorphonuclear cells and some edema. The cells were situated mainly in the interstitial tissue and particularly around the bronchi. Some, however, were present in the alveoli. This type of reaction was observed as late as 48 hours after inoculation, at which time the polymorphonuclear elements began to decrease in number. The walls of the alveoli and the tissues surrounding the bronchi and bronchioles then became infiltrated with lymphocytes and monocytes. In addition to the presence of these cells, the walls of the alveoli were definitely thickened with cuboidal cells which had vesicular nuclei and scanty, pale staining cytoplasm. It was impossible to determine the exact nature of these cells, but it is our impression that they were epithelial cells. Mitotic figures were seen in many of the cells present. The reaction observed 2 weeks after injection of the toxin and after the debris of the early polymorphonuclear reaction had been cleared away was mainly one of proliferation of the cells of the alveolar walls. It is thought that complete resolution would occur as no fibrous tissue was seen.

When large amounts of toxin were injected, the lesions became hemorrhagic and necrotic with polymorphonuclear cells infiltrating the necrotic areas.

In both types, particularly in the hemorrhagic form, some poly-



morphonuclear involvement of the arteries with, in certain instances, thrombus formation was found. The necrotic areas resemble infarcts, but it is thought that the necrosis occurred simultaneously with the vascular changes and not subsequent to them. This type of lesion never went on to complete resolution but always left scar tissue in the place of the necrosis.

#### DISCUSSION

Before we can say that the pneumonia described above is the result of the injected toxin, the possibility that the lesions were due to any of the following three causes should be excluded: First, that they were produced by bacteria. The sterility of the aerobic and anaerobic cultures of the lungs and the absence of bacteria in the microscopic preparations adequately refute this possibility. Second, that the toxin acted as a stimulus to an unrecognized virus lying dormant in the tissues. This possibility is excluded by the absence of inclusion bodies in the microscopic preparations, together with the failure to pass the disease to other rabbits. Third, that the lesions were non-specific. This assumption may be refuted by the absence of lesions when toxin-antitoxin mixtures were used and the marked reduction of the extent of the lesions when toxoid or heated toxin was used. Additional evidence that the lesions were not non-specific is the absence of lesions following the injection of a material similar to the *Staphylococcus aureus* toxin except that a *Staphylococcus albus* was employed in its preparation. Furthermore, although active immunization of rabbits by multiple subcutaneous injections of *Staphylococcus aureus* toxin failed to produce complete protection of the lungs, such treatments did decrease appreciably the intensity of the reaction. Essentially similar results were obtained in the animals that received large injections of the antitoxin intravenously the day before the intratracheal inoculation of the toxin was made. In the latter experiment, however, the intensity of the lesions bore an inverse ratio to the amount of antitoxin injected. In the light of the above experiments it is obvious that small doses of *Staphylococcus aureus* toxin and, to a lesser degree, diphtheria and streptococcus toxins, are capable of producing a mononuclear and proliferative pneumonia; whereas large doses of *Staphylococcus aureus* toxin cause an edematous, hemorrhagic,

and necrotizing pneumonia. This reaction is similar to that obtained by Muckenfuss and his associates (5) with vaccine virus. The only difference in the two sets of experiments is that in ours a large number of polymorphonuclear cells were present in the first 48 hours, whereas in the vaccine virus experiments only a few were seen. It is not unlikely that these cells appeared in response to some impurity in the preparation used and not to the toxin itself, inasmuch as the same amount of polymorphonuclear response was obtained with *Staphylococcus albus* preparations. It is thought that this type of response was due to the presence either of disintegrated bacteria or of substances in the culture medium.

The mononuclear proliferative pneumonia described by us is also similar to psittacosis pneumonia as seen in man and monkey and to the pneumonia complicating pertussis.

In view of these experiments we feel justified in maintaining that the proliferative and mononuclear portion of interstitial bronchopneumonia may be caused by bacterial toxins as well as by viruses.

We wish to call attention to the similarity of the pneumonias produced by toxins and viruses to that obtained by Winternitz and his associates (16) with acids and gases. Their results differed from ours in certain respects; *i.e.*, the epithelium of the bronchi and bronchioles was stimulated to a greater extent than in our experiments. Such facts suggest the possibility that this type of response of the lungs will result from any form of injurious agent that damages the cells without killing them. The injurious agent acts as a stimulus to cell growth and brings about a proliferation of the cells. However, if the amount of the agent is sufficient to cause the death of the tissue, a polymorphonuclear response is elicited by the dead tissue. In current experiments to be reported at a later date, this point and others dealing with the effect of combinations of bacteria with toxins are being investigated.

#### CONCLUSIONS

Bacterial toxins can produce a pneumonia similar to that caused by viruses, and the presence of a toxin-producing bacterium in the lungs may account for some of the interstitial bronchopneumonias observed in diseases other than those caused by viruses.

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## EXPLANATION OF PLATES

## PLATE 5

FIG. 1. Section of rabbit lung 72 hours after intratracheal inoculation of *Staphylococcus aureus* toxin. A zone of infiltration surrounds the bronchus, and the alveolar walls are thickened. The lumina of the bronchi are clear.  $\times 165$ .

FIG. 2. Same as Fig. 1 but showing a more intense reaction. The alveoli are lined with cells resembling the lining of the bronchi.  $\times 165$ .

FIG. 3. High power of Fig. 1 showing types of cells found in the alveoli. Note absence of polymorphonuclear cells.  $\times 730$ .

## PLATE 6

FIG. 4. Section of rabbit lung 72 hours after intratracheal inoculation of *Staphylococcus aureus* toxin, showing marked mononuclear periarteritis.  $\times 250$ .

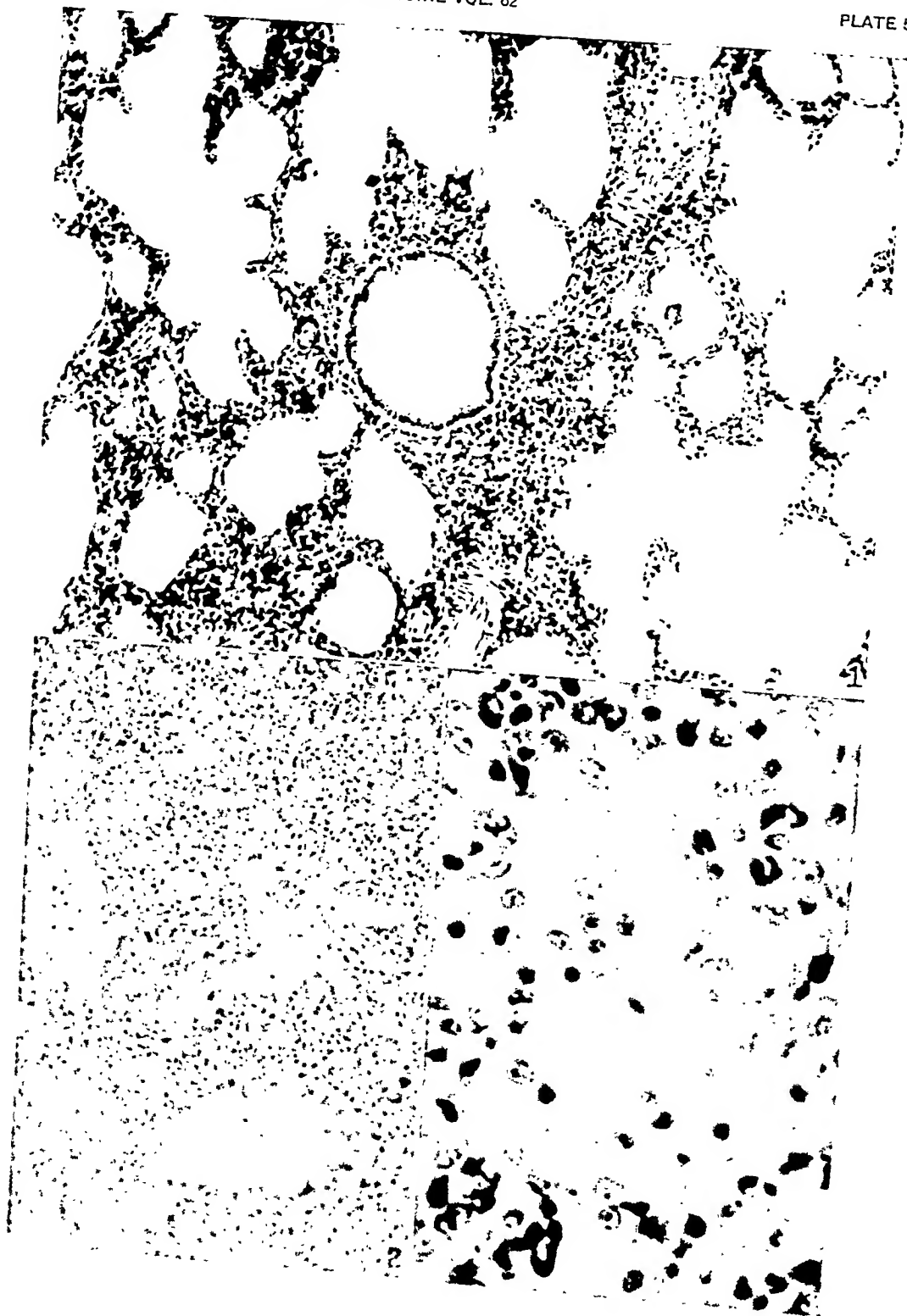
FIG. 5. Same as Fig. 4 but showing marked proliferation of cells in alveolar walls.  $\times 250$ .

FIG. 6. Higher power of Fig. 14 showing mitotic figures.  $\times 730$ .

PLATE 7

- FIG. 7. Section of rabbit lung 14 days after intratracheal inoculation of *Staphylococcus aureus* toxin. Note the syncytial-like mass of cells.  $\times 165$ .
- FIG. 8. Section of rabbit lung 72 hours after intratracheal injection of *Staphylococcus aureus* toxin. Note necrosis on the left. The bronchus is denuded of epithelium except in one place where there is proliferation.  $\times 98$ .







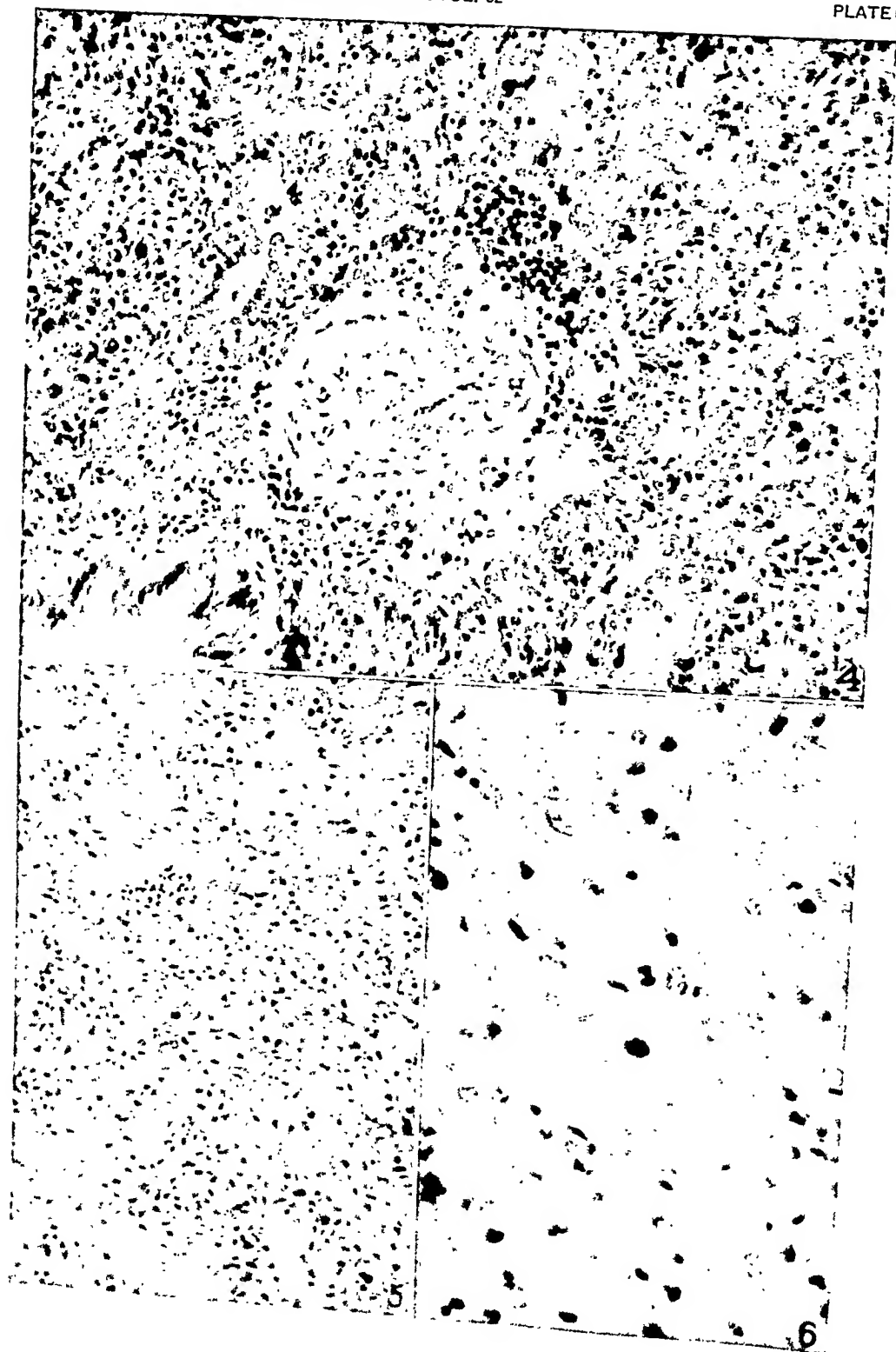








Fig. 1. (Continued from page 61)



# EXPERIMENTS ON THE EPIDEMIOLOGY OF PSEUDORABIES

## I. MODE OF TRANSMISSION OF THE DISEASE IN SWINE AND THEIR POSSIBLE RÔLE IN ITS SPREAD TO CATTLE

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The means by which pseudorabies is transmitted to cattle under natural conditions have never been determined. There is no evidence to suggest that the disease in this species is contagious or that ectoparasites play any rôle in transmitting the causative virus. In herds in which pseudorabies appears, it is a rule that only a relatively small proportion of the animals are affected; usually the morbidity rate does not exceed 10 per cent. Occasionally, as in the very first outbreak of the disease observed by the writer (1), it may be higher. Since this particular outbreak furnished an ideal field experiment to test the communicability of pseudorabies in cattle it will be described briefly at this time.

In August of 1930 an outbreak of pseudorabies in cattle was observed on a farm in Johnson County, Iowa. On this farm were two groups of cattle; 12 head of milk cows kept in the barn and barnlot and about 25 head of heifers and young steers kept in a nearby pasture. On Aug. 10 symptoms of pseudorabies appeared almost simultaneously in 4 of the milk cows. Two of these died during the night of Aug. 11 and the other 2 were killed when moribund on the 12th. Two new cases appeared among the milk cows on Aug. 12. For some unknown reason, the owner, on Aug. 12, turned all of the remaining milk cows, including 2 showing early symptoms of pseudorabies, into the same pasture with the 25 head of young stock. All animals drank from the same watering trough. The milk cows were returned to the barnlot each night while the young stock remained in the pasture. By Aug. 19, 2 more of the milk cows had developed the disease and had either died or been slaughtered when moribund. On Aug. 20 the 9th milk cow showed early symptoms of pseudorabies. It died on Aug. 22. After this no new cases appeared. The 5 remaining milk cows had at no time shown symptoms of pseudorabies and

the 25 head of young stock, in spite of rather intimate contact with 5 fatal cases of pseudorabies in the milk cows, failed to acquire the disease. This fortuitous experiment had rather clearly demonstrated that pseudorabies in cattle is not a communicable disease.

Nothing definite was learned regarding the mode of infection of the milk cows on this farm. That the animals had been infected while in the barn or barnlot was apparent. No new live stock had been recently purchased or brought to the farm. The entire herd, including the heifers in the pasture, had but recently recovered from a vulvitis. The owner stated that up to a week before the onset of pseudorabies there had been rats about the barnlot and that shortly before the first cases appeared he had removed several drowned rats from the watering trough in the barnlot. No other rat carcasses had been found and no live rats were seen about the farm while the disease was in progress. As on most middle western farms, there were numerous swine about the barnlot and these mingled intimately with the milk cows. None of the hogs had been observed to be ill, however, and none had died. The cattle, both in the barnlot and pasture, were being badly bitten by blood-sucking flies. The salient difference then between the young stock in which no cases of pseudorabies appeared and the milk cows of which 9 out of 12 succumbed to pseudorabies was that the former were on pasture and had no access to the barnlot in which the latter were kept. The only apparent potential animal sources of infection for the cattle in the barnlot were rats and swine. No rat carcasses were available for examination and the swine were not at the time suspected of playing a rôle in spreading pseudorabies virus to cattle.

Among the earlier workers with the disease, both Schmiedhoffer (2) and Hutyra (3) had suspected that rats played some rôle in the transmission of pseudorabies to cattle. The latter had been able to demonstrate pseudorabies virus in the brains of dead rats found on farms where pseudorabies was appearing in the cattle. More recently, Burggraaf and Lourens (4) have suspected that rats were of epidemiologic importance in an outbreak of pseudorabies observed by them in Holland.

Pseudorabies in rabbits, guinea pigs, white rats, and mice, like the disease in cattle, is not contagious. The writer has, on numerous occasions, placed normal animals of the above species in the same cage with those infected with pseudorabies and in not a single instance has an exposed animal acquired the infection. This finding is in agreement with the observations of others (5-7).

Experiments with pseudorabies in swine have supplied information suggesting that the disease in this species may be of epidemiological importance in the spread of the infection to cattle. It is the purpose of this paper to give an account of these experiments.

*Pseudorabies in Swine*

The susceptibility of swine to pseudorabies was not extensively investigated by earlier workers. Schmiedhoffer (2) was unable to infect pigs experimentally. On the other hand, von Rátz (8) observed the disease occurring naturally in both wild and domestic swine. In 1922 Manniger, according to Köves and Hirt (13), saw the disease in swine and noted the recovery of an animal that had shown symptoms of central nervous system involvement. Shope (1), using a strain of pseudorabies virus obtained from a cow in Iowa (mad itch), found that this virus administered subcutaneously produced in swine a mild disease in marked contrast to the uniformly fatal form which it produced in other species. In view of Schmiedhoffer's earlier unsuccessful attempts to infect swine experimentally, it was at first thought possible that the Iowa strain virus might differ materially from strains prevalent in Europe with regard to its pathogenicity for swine. Consequently a Hungarian strain of the virus was obtained from the late Professor Aládar Aujeszký. This Hungarian strain was found also capable of producing a mild non-fatal disease in swine when administered subcutaneously. Furthermore, sera of swine recovered from pseudorabies induced by either the Iowa or Hungarian strains of the virus were found capable of neutralizing both strains, thus demonstrating the immunological identity of the Hungarian and Iowa viruses (9).

More recently other investigators have noted the susceptibility of swine to experimental infection with pseudorabies virus (4, 10-13) and the disease has been recognized occurring naturally as a herd infection in this species in Holland (4) and Hungary (13).

Both the experimental and naturally occurring cases have been, in general, mild, and recovery of the infected swine after a transient illness has been the rule. In the outbreak studied by Burggraaf and Lourens (4) in Holland between 600 and 700 swine were affected. The illness observed was stated to be very slight and nearly all of the animals recovered after having shown very indistinct and transient symptoms. Köves and Hirt (13) have recently described swine pseudorabies as a rather common and apparently widespread infection in Hungary and an excellent account of the naturally occurring disease is given in their paper. In the large majority of the outbreaks observed by them the disease was not of a serious nature and the death loss was slight, ranging from 0 to 2 per cent but occasionally reaching as much as 5 per cent. The higher death losses were observed to occur during the winter months in swine that were being fattened.

Of greatest importance to the present investigation was the observation by Köves and Hirt that pseudorabies in swine is a contagious disease. They found that normal swine placed in the same pens with infected swine developed pseudorabies after an incubation period of 5 days or longer. They recorded no experiments to determine the mode of transmission of the virus from sick to normal hogs but suspected that it left the bodies of infected animals in the saliva, bronchial secretions, and urine and entered normal animals either through skin abrasions or by way of the alimentary canal. Shope (14) in a preliminary publication con-

firmed Köves and Hirt's observation that pseudorabies in swine is a communicable disease and cited experiments indicating that the nose served both as the portal of entrance and exit for the virus. These experiments will be outlined in more detail in the present paper.

### *Routes of Infection and Character of Disease Studied*

Pseudorabies virus has been found capable of infecting swine when administered subcutaneously, intramuscularly, intranasally, intracerebrally, or when fed. Fatal infections have been regularly produced in the intracerebrally inoculated swine, death occurring in from 36 to 96 hours. Administration of virus by all other routes has resulted in a more or less mild disease and, among 44 swine infected by ways other than intracerebral, no fatal cases have resulted or no animal, sacrificed before convalescence, has been seriously ill when killed. The salient clinical features of swine pseudorabies induced by subcutaneous inoculation are a 1 to 8 day febrile reaction during which the animals exhibit a transient depression and inappetence; rarely indefinite symptoms of central nervous system involvement were observed. Infection by routes other than subcutaneous results in a similar clinical picture but with minor variations. One out of 2 animals inoculated intranasally developed a pneumonia. Swine can be infected by feeding. A single feeding of virus failed to infect 1 animal, while repeated daily feedings for periods of from 4 to 7 days produced a disease in 3 swine that was identical in all respects with that following subcutaneous infection. Swine to which virus was administered intramuscularly in the ham or deep in the axillary space developed on the 3rd to 5th day following infection a transient or permanent flaccid paralysis of the inoculated leg. Aside from the paralysis, the disease seen in such swine was similar to that exhibited by subcutaneously infected animals.

Pseudorabies is a relatively highly contagious disease in swine, all things considered. In a series of 14 experiments normal swine were placed in the same isolation pen with pigs experimentally infected either subcutaneously or intramuscularly with pseudorabies virus. In 12 of these experiments the exposed swine developed pseudorabies. In 1 negative experiment it is believed, from what is known now, that the infected pig was removed before it had reached the stage at which the disease was communicable. The 2nd experiment in which an

exposed animal failed to develop pseudorabies will be referred to later. Although the Hungarian virus was used in most of these experiments, the Iowa strain (mad itch) was also found capable of transferring from pig to pig by contact.

Pseudorabies in swine infected by pen exposure was similar in all respects to the disease seen in pigs inoculated subcutaneously. None of the experimental animals died and none appeared dangerously ill. The nature of the illness contracted by exposure was proven in every case either by the demonstration of virus during the course of the disease or of specific virus-neutralizing antibodies in the blood serum following recovery. The latent period between the time of first exposure and the onset of symptoms varied from 3 to 11 days, depending upon the stage of the disease in the inoculated animal when the normal animal was placed in the pen.

#### *Mode of Transmission of Pseudorabies in Swine*

In studying the mode of transmission of pseudorabies in swine, it was found that hog lice played no rôle; using deloused or louse-free animals virus transferred readily from swine to swine on pen exposure. Neither could virus be demonstrated in the urine, feces, or salivary glands of infected pigs, indicating that the saliva and the excreta were unimportant as regards transmission.

Study of the distribution of the virus in swine killed early in an illness contracted by exposure finally suggested its portal of entrance. In such animals virus could be demonstrated only in the nasal washings. To test the possibility that virus might be spread from pig to pig by way of the nose the experiments recorded in Table I were conducted.

In these experiments the nasal passages of intramuscularly inoculated swine, as well as of those being exposed, were washed out daily with sterile physiological salt solution. A soft rubber catheter (size 10—French) was inserted 3 to 5 cm. into the anterior nares, with care not to abrade the mucosa, and approximately 30 cc. of the salt solution was forced into each nares by means of a half ounce irrigating syringe with rubber bulb. The wash fluid flowing from the nares was collected in a sterile wide mouthed bottle held below the animal's nose. From 50 to 75 per cent of the fluid used in the irrigation was recovered in most cases. Care was taken not to force the wash fluid so far into the nose as to permit the animal to swallow it. The washings were thus obtained largely from the anterior portion



of the swine's nasal passages. The wash fluid recovered always contained masses and strands of mucus and sometimes particles of food. The presence or absence of pseudorabies virus in the nasal washings was determined by injecting them in 2 cc. amounts subcutaneously into rabbits. Rabbits receiving washings that contained pseudorabies virus died showing the typical clinical picture of the disease (1) in from 3 to 7 days, depending upon the concentration of the virus contained in the washings.

As can be seen from Table I, virus was present in the nasal passages of the inoculated swine (1469 and 1483) on the 6th and 7th day following infection in the case of one animal, and on the 6th and 8th day in the case of the other. This was just at the end of the period of illness for both animals. Virus was present in the nasal washings of the swine infected by exposure on the day prior to the elevation of temperature above 40°C.; that is, on the last day of the period of incubation, and thereafter throughout the period of illness. The nasal washings of Swine 1482, under observation for 3 weeks, contained virus on the last day of the incubation period, on all the 6 days that the animal was ill, and on the first 4 days of convalescence. The blood, brain, spleen, and lung of Swine 1466, killed after its nasal washings had contained virus for 4 consecutive days, were free of virus as tested by subcutaneous inoculation into rabbits. The brain, blood, spleen, urine, and feces of Swine 1465, an exposure-infected swine not included in Table I, were free of pseudorabies virus when the animal was killed on the 3rd day of temperature elevation. Virus was abundantly present in the nasal washings of this animal. Virus was present in the lung and in a mixture of spleen and liver from Swine 1477, however, killed after its nasal washings had contained virus for 6 consecutive days. Urine and feces, blood, brain, and the salivary glands from this animal were free of virus.

In the intramuscularly infected swine the 6 day period elapsing between inoculation and the appearance of virus in the nose is believed to represent the time required for the virus to spread from the site of inoculation to the nasal mucous membranes. Experiments in which the distribution of virus in the bodies of inoculated swine has been studied suggest that its spread from the site of inoculation to the nose takes place largely by way of the peripheral and central nervous system. Hurst's experiments (15) demonstrated that pseudorabies virus behaved like a true neurotrope in laboratory animals in that its spread

TABLE I  
*The Presence of Pseudorabies Virus in the Nasal Washings of Infected Swine*

|                                 | Experiment 1                                   |                                 | Experiment 2                                   |                                 |                                 |
|---------------------------------|--|---------------------------------|--|---------------------------------|---------------------------------|
|                                 | Swine 1469 infected with virus intramuscularly | Swine 1466 infected by exposure | Swine 1483 infected with virus intramuscularly | Swine 1482 infected by exposure | Swine 1477 infected by exposure |
| 1                               | —  | Placed in pen with ← Swine 1469 | 0  | Placed in pen with ← Swine 1483 |                                 |
| 2                               | X  | 0                               | X  | 0                               |                                 |
| 3                               | X  | 0                               | X  | —                               |                                 |
| 4                               | X  | 0                               | X  | —                               |                                 |
| 5                               | X  | 0                               | X  | 0                               |                                 |
| 6                               | X  | 0                               | X  | —                               |                                 |
| 7                               | X + (101 hrs.)                                 | 0                               | X + (135 hrs.)                                 | —                               |                                 |
| 8                               | + (117 " )                                     | 0                               | X  | 0                               |                                 |
| 9                               | —  | 0                               | —  | —                               |                                 |
| 10                              | —  | 0                               | —  | —                               |                                 |
| 11                              | —  | 0                               | —  | —                               |                                 |
| 12                              | —  | 0                               | —  | —                               |                                 |
| 13                              | —  | 0                               | —  | —                               |                                 |
| 14                              | —  | + (166 hrs.)                    | —  | —                               |                                 |
| 15                              | —  | X + (98 " )                     | —  | —                               |                                 |
| 16                              | —  | + (79 " )                       | —  | —                               |                                 |
| 17                              | —  | + (82 " )                       | —  | —                               |                                 |
| 18                              |  |                                 |  |                                 |                                 |
| 19                              |  |                                 |  |                                 |                                 |
| 20                              |  |                                 |  |                                 |                                 |
| 21                              |  |                                 |  |                                 |                                 |
| 22                              |  |                                 |  |                                 |                                 |
| Placed in pen with ← Swine 1482 |  |                                 |  |                                 |                                 |
|                                 |  |                                 |  |                                 | 0                               |
|                                 |  |                                 |  |                                 | —                               |
|                                 |  |                                 |  |                                 | + (126 hrs.)                    |
|                                 |  |                                 |  |                                 | X + (95 " )                     |
|                                 |  |                                 |  |                                 | X + (101 " )                    |
|                                 |  |                                 |  |                                 | X + (85 " )                     |
|                                 |  |                                 |  |                                 | X + (71 " )                     |
|                                 |  |                                 |  |                                 | X + (63 " )                     |

X = temperature elevation to fever level (40°C. or higher).  
 0 = nasal passages not lavaged.  
 — = virus absent—test rabbit survived.  
 + = virus present—test rabbit died of pseudorabies. (Hours in parentheses indicate time elapsing between inoculation and death.)

from the site of inoculation took place largely by neural routes. Conversely, in swine infected by exposure the nose contains virus for at least 4 days before any can be shown to have spread to other parts of the body.

The experiments outlined in Table I and just summarized indicate that in pseudorabies in swine the nose serves both as the portal of entrance and as an exit of the virus.

*The Transmission of Pseudorabies by Contact from the Nose of Swine to the Abraded Skin of Rabbits*

The preceding experiments, dealing with the mode of transmission of pseudorabies from swine to swine as a communicable disease, suggested that this species might serve as an important reservoir for the virus. From such a swine reservoir the virus could conceivably escape at opportune times to cause the sporadic and highly fatal cases of pseudorabies seen in cattle in the swine-raising states of the middle West. A number of known facts concerning the disease make this possibility seem likely. Pseudorabies in swine is highly contagious, it is not fatal for this species among all of its hosts, and it is often of so mild and ill defined a nature that it may escape notice, and, as will be shown later, has done so in this country. The hog can be a potential source of infection for cattle and not be suspected; an almost "silent" host. Furthermore, in sections of this country where pseudorabies in cattle is endemic it is a common practice for the farmer to keep his cattle and swine together in the same enclosures.

In 6 outbreaks of pseudorabies in cattle which were either observed personally or concerning which direct information was available, swine and cattle were confined in the same pens in every instance. In one of these outbreaks pooled blood serum was obtained from swine that had associated with the cattle. It was capable of neutralizing pseudorabies virus, indicating that the hogs furnishing it were convalescent from the disease. No illness had been noted in the swine. It is suggested that the swine infection may have preceded the bovine infection and that the virus spread from the hogs to the cattle. In another outbreak 5 out of 40 head of cattle that were being fed in the barnlot with a large group of swine developed pseudorabies and either died or were sacrificed when moribund. No illness was noted in the swine. A month after

the death of the last cow, blood serum was obtained from 6 swine and 6 of the surviving cattle. None of the 6 cattle sera neutralized pseudorabies virus; whereas, 4 out of the 6 swine sera proved virus-neutralizing. The results with the cattle sera indicate the probability that all of the cattle infected with the virus died and that none of the survivors underwent a subclinical and unrecognized infection. They thus support further the view that pseudorabies is not a communicable disease among cattle. The findings with swine sera indicate a fairly high previous pseudorabies infection among the hogs with which the cattle associated. It is believed that the swine on this farm may have been responsible for the disease in the cattle.<sup>1</sup>

Dr. Ortiz Patto of Bello Horizonte, Brazil, in a personal communication, has informed me that in all of the outbreaks of bovine pseudorabies seen by him, the infected cattle had been kept in intimate contact with swine. Burggraaf and Lourens (4) have stated that in an outbreak of pseudorabies in Holland in 1932, in which the disease was recognized as a relatively mild illness in swine, but highly fatal in cattle, the swine became ill before the cattle on all farms. These authors further noted that with the exception of 4 cows on two farms, all cattle which became infected had been housed in the same buildings with sick hogs. Burggraaf and Lourens, however, were unable to demonstrate that pseudorabies either in cattle or in swine was a contagious disease.

To one familiar with the behavior of swine when they are with cattle, it seems likely that a virus present in the nose of a hog, or more especially on the nose, could be transferred to the skin of a cow. Cattle lying about a barnlot in which hogs are also kept come frequently in contact with the pigs' noses. Swine under such conditions can be observed to approach a cow and probe it in the flank or side with their noses. If the first punch fails to get the cow up, a second and more vigorous probe is given. This process is continued more persistently, and not infrequently, as a last resort, they will bite the cow, often through the areas of skin probed with their noses.

To determine whether pseudorabies virus could transfer from the noses of infected swine to the abraded skin of susceptible animals, experiments were conducted in which, instead of lavaging the hog's nasal passages with saline to obtain virus as had been done in the experiments recorded in Table I, their noses were merely rubbed against the freshly abraded abdominal skin of rabbits. The bellies of rabbits to be used in such experiments were shaven a day early to insure leaving a dry snap free skin surface. The skin of the rabbits was scarified by cross-hatching with a curved cutting surgical needle deeply enough to cause oozing of

<sup>1</sup> I am indebted to Dr. C. H. Banks of Tipton, Iowa, for calling my attention to these two outbreaks and for obtaining the serum samples studied.

blood-tinged plasma along portions of the scarifications. These freshly scarified areas were then immediately rubbed against the noses of infected swine held by an attendant.

The results of one such experiment in which the disease was passed by exposure serially through 4 swine are recorded in Table II.

The experiment outlined in Table II indicates that pseudorabies virus can pass from the nose of infected swine to the abraded skin of susceptible hosts by direct contact. They further suggest that there is considerable individual variation in the ability of different swine to transfer the infection in this way. For instance, Swine 1495, infected by exposure, transferred virus to rabbits throughout the entire course of its illness. In like manner, Swine 1493, infected with pseudorabies by inoculation, transmitted the disease to rabbits on the 6th and 7th day following infection as might be anticipated from the results obtained with inoculated swine recorded in Table I. However, the remaining 3 swine in the experiment infected rabbits only irregularly and occasionally. There is no reason to doubt the presence of pseudorabies virus in the noses of these animals throughout the course of their disease; it has been so readily and constantly demonstrable in the nasal washings of other similarly infected swine. Their failure to infect on all expected occasions would seem rather to be due either to the absence of pseudorabies virus on the surface of the noses, as contrasted with its presence within the nasal passages, or to the greater resistance of the test rabbits to infection by sacrifice than to infection by subcutaneous inoculation with washings. That the first possibility may be the correct one is suggested by the experience with Swine 1503. Virus had been demonstrable on the surface of the nose of this particular hog, as shown in Table II, only on the 2nd day of fever. To judge from the results recorded in the table, it had not been present for the next 5 days. However, nasal washings obtained at autopsy at the end of this 5 day period during which the surface of the nose had apparently been free of virus, killed a rabbit typically of pseudorabies when administered subcutaneously. Lavaging the noses of swine recorded in Table II was purposely avoided for fear of defeating the primary purpose of this experiment; that of demonstrating that virus could transfer from the nose of swine to the abraded skin of rabbits. It was conceivable that lavage might wash virus from the

nasal passages and deposit it on the surface of the nose. This would have influenced the outcome of the experiment and given a false impression of the readiness with which virus could spread from the nose of swine to the abraded skin of rabbits. There is no apparent way of determining whether the rabbits in Experiment 2 that failed to become infected with pseudorabies, as anticipated from consideration of the experiments in Table I, did so because of the absence of virus on the surface of the infected pig's nose, or because the test for the presence of virus in Experiment 2 was less delicate than that employed in Experiment 1.

The experiments given in Table II, in spite of the gaps in the record of infections produced, are interpreted as indicating that pseudorabies virus can spread from the nose of swine to another host by way of the abraded skin. Furthermore, it is believed that under farmyard conditions, cattle can acquire pseudorabies by such contact with infected hogs.

#### DISCUSSION

Experiments with pseudorabies in swine have suggested a possible explanation of the means by which the disease is transmitted to cattle under natural conditions. Experimental infection of swine with the virus results in an illness that is both mild and ill defined. Furthermore, porcine pseudorabies, in contrast to pseudorabies in cattle or any of the small laboratory animals, is a highly contagious disease relatively speaking. Evidence that the nose serves both for the entrance and the exit of the virus has been presented. In swine infected by inoculation, virus is not demonstrable in nasal washings until the very end of the disease. In swine infected by exposure to inoculated animals, however, virus may be demonstrable in the nose on the last day of the period of incubation, throughout the period of illness, and for several days after the animal's temperature has returned to normal. Such animals thus are potential sources of infection for a number of days. During the early part of a pseudorabies infection induced by exposure the virus can be shown to be present only in the nose, while later on it may spread to the lung, brain, spinal cord, or spleen.

Out of a group of 14 experiments in which normal swine were exposed

TABLE II  
*The Presence of Pseudorabies Virus on the Noses of Infected Swine as Demonstrated by the Direct Infection of Rabbits*

| Day of experiment | Swine 1493 infected with virus subcutaneously | Swine 1509 infected by exposure | Swine 1495 infected by exposure | Swine 1503 infected by exposure | Swine 1515 infected by exposure |
|-------------------|---|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| 1                 | 0   | Placed in pen with ←Swine 1493  |                                 |                                 |                                 |
| 2                 | X   | 0                               |                                 |                                 |                                 |
| 3                 | X   | 0                               |                                 |                                 |                                 |
| 4                 | X   | —                               |                                 |                                 |                                 |
| 5                 | X   | —                               |                                 |                                 |                                 |
| 6                 | X + ( 87 hrs.)                                | —                               |                                 |                                 |                                 |
| 7                 | + (118 " )                                    | —                               |                                 |                                 |                                 |
| 8                 | —   | X                               | Placed in pen with ←Swine 1509  |                                 |                                 |
| 9                 | 0   | X                               | 0                               |                                 |                                 |
| 10                | —   | X                               | 0                               |                                 |                                 |
| 11                | —   | X                               | + ( 94 hrs.)                    |                                 |                                 |
| 12                | —   | X                               | + ( 89 " )                      |                                 |                                 |
| 13                | —   | X                               | X + ( 82 " )                    |                                 |                                 |
| 14                | —   | X                               | X + ( 79 " )                    |                                 |                                 |
| 15                | —   | X                               | X + ( 83 " )                    | Placed in pen with ←Swine 1495  |                                 |
| 16                |   | K + (105 hrs.)                  | X + ( 82 " )                    | 0                               |                                 |
| 17                |   |                                 | X + ( 91 " )                    | —                               |                                 |
| 18                |   |                                 | X + ( 80 " )                    | —                               |                                 |
| 19                |   |                                 | X                               | —                               |                                 |
| 20                |   |                                 | X + (118 " )                    | X                               |                                 |
| 21                |   |                                 | —                               | X + (94 hrs.)                   |                                 |
| 22                |   |                                 | —                               | X                               |                                 |

K → killed and the following distribution of pseudorabies virus determined: Swine 1509—virus present in spleen. No virus demonstrable in heart blood, liver, lung, or salivary glands. Swine 1503—virus present in nasal washings. No virus present in brain and upper dorsal cord. No virus demonstrable in heart blood, nasal turbinates, spleen, kidneys, liver, salivary glands, cervical and mesenteric lymph nodes, brain, or lung. Swine 1515—virus present in heart blood, spleen, liver, salivary glands, cervical and mesenteric lymph nodes, or lung.



to infected animals, the virus transferred to the exposed pigs in all but two instances. In one of these negative experiments it is believed from what is known now that the infected pig was removed from the exposure pen before it had reached a stage at which its disease was communicable. The other negative experiment was of interest because it was the only one in which no virus could be demonstrated in the nasal washings of the infected swine. It is believed that the disease failed to transfer from the sick to the normal animal in this experiment because the virus for some unknown reason did not reach the nose of the infected animal.

Experiments in which the noses of infected swine have been rubbed against the scarified skin of rabbits have shown that the virus can transfer from infected swine to a second susceptible host in this manner. It is believed that under farm conditions in which swine are kept in intimate contact with cattle, cattle may be infected in this way. On two farms where cattle had died of pseudorabies the sera of swine with which the cattle had associated have been found to contain pseudorabies virus-neutralizing antibodies. This observation has been interpreted as indicating that these swine had an unrecognized pseudorabies infection and that they may have served as the source from which virus causing the fatal bovine infections was acquired.

#### SUMMARY

Pseudorabies is a very fatal but non-contagious disease in cattle and the common laboratory animals. It is a relatively mild yet highly contagious disease in swine. It has been shown that in swine the nose serves both for the entrance and the exit of the virus. Furthermore, it has been observed that fatal pseudorabies infections in rabbits can be induced merely by bringing their abraded skin into contact with the noses of infected swine. The blood sera of swine on two farms where pseudorabies had occurred among the cattle were found to be capable of neutralizing pseudorabies virus. It is believed that in these instances the swine had a mild and unrecognized pseudorabies infection and transmitted their disease to the cattle with which they were associated, by transfer of the virus on their noses to the abraded skin of the cattle.

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## EXPERIMENTS ON THE EPIDEMIOLOGY OF PSEUDORABIES

### II. PREVALENCE OF THE DISEASE AMONG MIDDLE WESTERN SWINE AND THE POSSIBLE RÔLE OF RATS IN HERD-TO-HERD INFECTIONS

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In the preceding paper (1) the mode of transmission of pseudorabies among swine was discussed and experimental evidence was presented that this species might serve as a source of infection for cattle. The possibility seemed reasonable enough from the laboratory findings. However, apart from the two examples mentioned in the preceding paper, there was no evidence to indicate that pseudorabies existed in our middle western swine population. The mild and indefinite nature of the disease in hogs has suggested the possibility that it might be prevalent but unrecognized in swine in the United States.

#### *I. Attempts to Determine the Prevalence of Pseudorabies among Middle Western Swine*

It is known from earlier work (2-5) that the blood serum of swine recovered from an attack of pseudorabies is specifically virus-neutralizing. The blood serum of normal swine or of swine immune to other diseases has been found by the writer to be incapable of neutralizing pseudorabies virus. It seemed certain, therefore, that the presence of pseudorabies virus-neutralizing antibodies in the blood serum of swine could be taken as a criterion of an earlier infection of the animals with pseudorabies. It has been utilized in the present studies to determine the prevalence of pseudorabies among middle western swine.

Preliminary experiments were conducted to determine the approximate virus-neutralizing activity of sera from swine recovered from experimental pseudorabies.

The neutralization tests were run in the usual fashion. The Hungarian (Aujeszky) strain of pseudorabies virus was used in all tests in preference to the Iowa strain (mad itch), because of its slightly greater and more constant virulence for guinea pigs. Each neutralization mixture was set up to contain 100 mg. of rabbit brain virus (1 cc. of a 10 per cent suspension of the brain of a rabbit dead following intracerebral infection). This amount of virus represented between 100 and 1000 minimal fatal doses for guinea pigs subcutaneously. The mixtures of serum and virus were stored in the refrigerator (+4°C.) overnight (17 hours) prior to injection subcutaneously into guinea pigs. Control mixtures contained the same amount of virus mixed with normal swine serum. In this and all subsequent neutralization experiments to be recorded, virus that had been stored in 50 per cent glycerol in the refrigerator for at least a week was used in preference to fresh unglycerolated virus.

The virus-neutralizing activity of 8 convalescent swine sera is recorded in Table I.

As shown by Table I, the virus-neutralizing titer of the sera of 6 of the swine was such that 0.05 cc. completely neutralized 100 mg. of pseudorabies virus, while 0.01 cc. failed to do so. The serum of one animal was active in an amount smaller than 0.01 cc., while 0.1 cc. of the serum of another neutralized virus although 0.05 cc. failed to do so. These figures will be utilized later in trying to determine the incidence of infection among groups of swine from which only pooled serum samples were available for testing.

#### *The Presence of Pseudorabies Virus-Neutralizing Antibodies in Commercial Anti-Hog Cholera Serum*

It was impractical to obtain individual blood serum samples from large numbers of swine in scattered mid-western communities for testing. Fortunately, however, for the purpose of these experiments, pooled serum samples from relatively large numbers of hogs were easily available in the form of commercial anti-hog cholera serum.

Briefly stated, anti-hog cholera serum is obtained from swine that have been hyperimmunized against hog cholera. It is collected in batches composed of 300 liters or less. These batches frequently contain all of the serum collected from approximately 50 hogs. All of the serum derived from these hogs is mixed in a single container and identified by number as a batch. In other cases these batches contain some serum derived from as many as 175 hogs. The blood of these hogs in such circumstances forms a part of 3 or 4 different batches, as previously described. Complete records of each serial number of commercially produced anti-

hog cholera serum, including the number of swine whose serum is represented therein, are maintained by the producer and the Bureau of Animal Industry of the United States Department of Agriculture.<sup>1</sup>

The availability of the above rather exact information as to composition made it appear profitable to study a number of samples of commercial anti-hog cholera sera to determine whether any contained virus-neutralizing antibodies effective against pseudorabies virus. From consideration of the experiments recorded in Table I, it was felt that the neutralization test as employed was sensitive enough to detect a virus-neutralizing serum even though it had been diluted 1 to 20 with non-virus-neutralizing sera. An incidence of infection lower than 5 per cent might not be detected by the neutralization technique employed.

TABLE I

*The Titer of Virus-Neutralizing Antibodies in the Blood Serum of Swine Recovered from Pseudorabies*

| Serum from swine No. | Mode of infection of swine | Serum drawn post-infection | Amount of swine serum mixed with 100 mg. of pseudorabies virus and administered subcutaneously to guinea pigs |              |              |
|----------------------|----------------------------|----------------------------|---|--------------|--------------|
|                      |                            |                            | 0.1 cc.   | 0.05 cc.     | 0.01 cc.     |
|                      |                            |                            | Result  | Result       | Result       |
|                      |                            | days                       |   |              |              |
| 1330                 | Exposure to infected swine | 27                         | No illness  | No illness   | Died—76 hrs. |
| 1334                 | Virus intranasally         | 13                         | " "   | " "          | " 82 "       |
| 1347                 | Virus fed                  | 19                         | " "   | " "          | " 163 "      |
| 1457                 | Virus intramuscularly      | 14                         | " "   | " "          | " 104 "      |
| 1469                 | " "                        | 57                         | No illness  | " "          | " 66 "       |
| 1482                 | Exposure to infected swine | 24                         | " "   | " "          | " 87 "       |
| 1493                 | Virus subcutaneously       | 35                         | " "   | " "          | No illness   |
| 1495                 | Exposure to infected swine | 20                         | " "   | Died—63 hrs. | Died—63 hrs. |

The control guinea pigs receiving 100 mg. of virus mixed with normal swine serum all succumbed typically of pseudorabies and are not recorded in this table.

The neutralization tests were run in the way already outlined for the experiments recorded in Table I. Each sample of anti-hog cholera serum was tested in two amounts as a rule, 1 cc. and 0.1 cc. Since the anti-hog cholera sera all contained 0.5 per cent phenol added as a preservative, a similar amount of phenol was added to the normal swine sera controlling the present experiments. All the samples of anti-hog cholera serum used were produced from middle western swine by middle western concerns, manufacturing veterinary biological materials. The

<sup>1</sup>I am indebted to Dr. D. L. Skidmore, Chief of the Division of Virus-Serum Control of the United States Bureau of Animal Industry for information concerning these results.

samples were purchased on the open market by thirteen veterinary practitioners in eastern Iowa.<sup>2</sup> The brands and serial numbers of sera tested are not recorded and the samples are designated only numerically. No duplicate samples are included in the experimental record which is given in Table II. All of the experiments outlined in this table were not conducted at the same time. Controls were, however, included with each group of experiments as they were done and are recorded compositely in the table.

The results obtained with the commercial anti-hog cholera serum were surprising. Of the 23 samples tested, only 2 (samples 9 and 17) failed to neutralize pseudorabies virus in the largest dosage employed. This is interpreted as meaning that the swine furnishing these 2 sera had either been entirely free of pseudorabies or that the incidence of the disease in this group had been less than 5 per cent. Of the remaining 21 samples, 9 neutralized virus in 1 cc. amounts but not in 0.1 amounts. This is interpreted as indicating an incidence of previous pseudorabies infection of 5 per cent or greater among the group of swine furnishing these sera. The remaining 12 serum samples neutralized pseudorabies virus in both 1.0 and 0.1 cc. amounts, indicating an incidence of previous pseudorabies infection of upwards of 50 per cent in the group of swine furnishing the sera. While it was realized that these data, at best, were only approximate, they indicated that porcine pseudorabies was indeed a prevalent disease.

It was difficult to believe that the figures obtained actually represented the incidence of pseudorabies infection among middle western swine used in the preparation of anti-hog cholera serum. The indication that a disease could be so prevalent and not be recognized was startling. The writer's first reaction was that the results obtained must have some other explanation. Several alternative explanations suggested themselves. These were investigated.

#### *Absence of an Immunological Relationship between the Viruses of Pseudorabies and Hog Cholera*

Although the likelihood of an immunological relationship between the viruses of pseudorabies and hog cholera seemed remote, the almost constant neutralization of the former virus by sera prepared commer-

<sup>2</sup> Drs. Banks, Bryant, Carey, Crow, Dunn, Glenn, Hawthorne, Lames, Morantville, Potter, Strader, Vollstedt, and Wilson kindly furnished me with these samples from sera that they were using in their swine practice.

**TABLE II**  
*The Neutralization of Pseudorabies Virus by Commercial Anti-Hog Cholera Sera*

| Anti-hog cholera serum sample | No. of swine represented in sample | Amount of serum mixed with 100 mg. of pseudorabies virus and administered subcutaneously to guinea pigs |              |
|-------------------------------|------------------------------------|---|--------------|
|                               |                                    | 1 cc.   | 0.1 cc.      |
|                               |                                    | Result  | Result       |
| 1                             | 50                                 | No illness  | Died—58 hrs. |
| 2                             | 146                                | " "   | No illness   |
| 3                             | 152                                | " "   | Died—95 hrs. |
| 4                             | 151                                | " "   | No illness   |
| 5                             | 150                                | " "   | " "          |
| 6                             | 148                                | " "   | Died—73 hrs. |
| 7                             | 48                                 | " "   | " 104 "      |
| 8                             | 158                                | " "   | No illness   |
| 9                             | 110                                | Died—95 hrs.  | No illness   |
| 10                            | 170                                | No illness  | No illness   |
| 11                            | 103                                | " "   | " "          |
| 12                            | 100                                | " "   | " "          |
| 13                            | 129                                | " "   | " "          |
| 14                            | 169                                | " "   | " "          |
| 15                            | 106                                | " "   | " "          |
| 16                            | 165                                | " "   | Died—70 hrs. |
| 17                            | ?                                  | " "   | " 91 "       |
| 18                            | ?                                  | Died—69 hrs.  | No illness   |
| 19                            | 85                                 | No illness  | Died—84 hrs. |
| 20                            | 152                                | " "   | " 68 "       |
| 21                            | 103                                | " "   | No illness   |
| 22                            | 126                                | " "   | " "          |
| 23                            | ?                                  | " "   | " "          |

**Control sera\***

| Swine 1237—Institute stock† |   |   | Died—68 hrs. | Died—63 hrs. |
|-----------------------------|---|---|--------------|--------------|
| "                           | " | " |              |              |
| " 1237                      | " | " | " 66 "       | " 61 "       |
| " 1237                      | " | " | " 52 "       | " 70 "       |
| " 1444                      | " | " | " 64 "       |              |
| " 1444                      | " | " | " 63 "       |              |
| " 1449                      | " | " | " 73 "       |              |
| " 1449                      | " | " | " 58 "       |              |
| " 1229                      | " | " | " 70 "       |              |
| " 1229                      | " | " | " 95 "       |              |
| " 1446                      | " | " | " 70 "       |              |
| " 1446                      | " | " | " 58 "       |              |
| " 1446                      | " | " | " 70 "       |              |

\* 0.5 per cent phenol was added to all control sera to make them comparable to the commercial anti-hog cholera sera. The phenol was added as follows: to 14 parts of serum, 1 part of an aqueous solution containing 7.5 per cent phenol and 10 per cent glycerol was added. This complies with the general method employed by commercial concerns in phenolizing their anti-hog cholera serum.

† Swine 1237 and 1444 were raised on the Institute farm and had had no known infectious disease. Swine 1449 and 1229 were from the same stock and had been hyperimmunized against hog cholera. Swine 1446, from the same stock, had been immunized but not hyperimmunized against hog cholera.



cially against the latter made it necessary to eliminate this possibility. This was easily done. As shown in Table II, serum from Swine 1449 and 1229, hyperimmunized against hog cholera in the usual fashion, failed to neutralize pseudorabies virus, indicating that the mere procedure of hog cholera hyperimmunization was not responsible for the appearance of pseudorabies virus-neutralizing antibodies in swine sera. Furthermore, other experiments showed that the immunization of swine against one of these viruses conferred no protection against infection with the other virus; swine that had been vaccinated against hog cholera and that were demonstrably immune to that virus were still susceptible to pseudorabies. In like manner, swine that had recovered from pseudorabies and that were demonstrably immune to that virus were still fully susceptible to hog cholera. It seems certain that hyperimmunization against hog cholera cannot, therefore, account for the high pseudorabies virus-neutralizing antibody content of the samples of commercial anti-hog cholera serum recorded in Table II.

*The Presence of Pseudorabies Virus-Neutralizing Antibodies in the Blood Serum of Individual Swine Used in the Commercial Production of Anti-Hog Cholera Serum*

Through the courtesy of Dr. B. M. Lyon, I was permitted to obtain blood serum from 15 hyperimmunized swine being bled at the Lederle Laboratories during the course of their routine production of anti-hog cholera serum. These animals had come originally from the middle West having been purchased in St. Paul, Minnesota. Their sera were tested against the standard 100 mg. dose of pseudorabies virus in 1.0 and 0.1 cc. amounts and the results obtained are recorded in Table III. No antiseptic had been added to these sera.

The sera from Swine L.S. 1195 and L.S. 1210 failed to neutralize pseudorabies virus even in 1 cc. amounts, indicating that these animals had suffered no earlier pseudorabies infection. The sera from the remaining 13 swine were all virus-neutralizing in 1 cc. amounts. This is interpreted as indicating that these 13 animals had undergone an earlier infection with pseudorabies virus. The results obtained when the 13 effective sera were tested in 0.1 cc. amounts were somewhat unexpected. It had been anticipated, from experience with known pseudorabies convalescent sera from experimentally infected swine (see Table I), that a serum found to be virus-neutralizing in

0.0 cc. amounts would also neutralize the standard dosage of virus in 0.1 cc. amounts. However, 4 of the 13 sera recorded in Table III

TABLE III  
*The Presence of Pseudorabies Virus-Neutralizing Antibodies in the Blood Serum of Individual Swine Hyperimmunized against Hog Cholera*

| Serum from swine No. | Amount of serum mixed with 100 mg. of pseudorabies virus and administered subcutaneously to guinea pigs |               |
|----------------------|---|---------------|
|                      | 1 cc.   | 0.1 cc.       |
|                      | Result  | Result        |
| L.S. 1193            | No illness  | No illness    |
| " " 1195             | Died—73 hrs.  | No illness    |
| " " 1196             | No illness  | No illness    |
| " " 1197             | " "   | Died—81 hrs.  |
| " " 1201             | " "   | No illness    |
| " " 1202             | " "   | Died—61 hrs.  |
| " " 1203             | " "   | No illness    |
| " " 1204             | " "   | " "           |
| " " 1205             | " "   | " "           |
| " " 1206             | " "   | Died—99 hrs.  |
| " " 1207             | " "   | No illness    |
| " " 1208             | " "   | " "           |
| " " 1209             | " "   | Died—107 hrs. |
| " " 1210             | " "   | No illness    |
| " " 1211             | Died—84 hrs.  | " "           |
|                      | No illness  |               |
| Control sera         |   |               |
| 1237*                | Died—56 hrs.  |               |
| 1237                 | " 66 "  |               |
| 1237                 | " 63 "  |               |
| 1444                 | " 61 "  |               |
| 1229                 | " 61 "  |               |
| 1439                 | " 81 "  |               |
| 1544                 | " 81 "  |               |
| 1545                 | " 72 "  |               |

\* Swine 1237 and 1444 were raised on the Institute farm and had had no known infectious disease. Swine 1229 and 1449 from the same stock had been hyperimmunized against hog cholera. Swine 1544 and 1545 were purchased from a nearby farmer and had been immunized against hog cholera.

failed to neutralize virus in 0.1 cc. amounts. This result suggests that in pseudorabies, as in other virus infections, serum from

recently recovered animals is richer in virus-neutralizing antibodies than that from animals recovered for longer periods of time. It seems likely that the virus-neutralizing titer of the serum rises to reach a peak sometime shortly after recovery and then gradually declines. On this basis, the low antiviral titer of 4 of the serum samples would suggest that the swine furnishing these particular sera had probably suffered their pseudorabies infection months earlier, perhaps when they were small pigs. The animals in this particular group weighed from 250 to 350 pounds and hence were approximately 1 year of age. Another possible explanation may be that some field strains of the pseudorabies virus are not capable of eliciting as high an antibody reaction as that achieved by the highly virulent Hungarian strain used in the present experiments.

On the basis of the possibility just suggested that the pseudorabies virus-neutralizing titer of swine serum can at times lie between 1.0 and 0.1 cc., instead of between 0.05 and 0.01 cc. as indicated by the results recorded in Table I, the estimates as to the incidence of previous pseudorabies infection among swine furnishing the pooled samples of commercial anti-hog cholera serum recorded in Table II may be too conservative. The finding that 13 out of 15 individual swine being used in the commercial production of anti-hog cholera serum had had an earlier pseudorabies infection, however, lends plausibility to the high figures for the incidence of the infection indicated by the experiments with the pooled sera.

*The Presence of Pseudorabies Virus-Neutralizing Antibodies in the Blood Serum of Middle Western Swine Killed at a Local Slaughterhouse*

Blood was obtained at the time of slaughter from 10 swine killed at a local slaughterhouse. These animals were part of a shipment said to have come from the middle West via the stockyards at Lancaster, Pennsylvania. No accurate information as to their true geographical origin was available. They weighed from 300 to 350 pounds and, to judge by the heterogeneity of breed, were probably from several different sources. The results of neutralization tests on the blood sera of these 10 swine are recorded in Table IV.

The sera from all 10 animals when tested in 1 cc. amounts neutralized the standard dose of pseudorabies virus. This is interpreted as indicating that these hogs had all undergone an earlier infection with pseudorabies virus. As in the case of some of the cholera hyperim-

mune swine recorded in Table III, the virus-neutralizing titer of some of the sera was low.

*Tests for the Presence of Antibodies for Pseudorabies in the Blood Serum of Large Local Swine*

The control sera used throughout the virus neutralization experiments recorded in this paper had been obtained largely from swine weighing 40 to 125 pounds.

TABLE IV  
*The Presence of Pseudorabies Virus-Neutralizing Antibodies in the Blood Serum of Middle Western Swine Killed at a Local Slaughterhouse*

| Serum from swine No. | Amount of serum mixed with 100 mg. of pseudorabies virus and administered subcutaneously to guinea pigs |              |              |
|----------------------|---|--------------|--------------|
|                      | 1 cc.   | 0.3 cc       | 0.1 cc.      |
|                      | Result  | Result       | Result       |
| T. 1                 | No illness  |              | No illness   |
| " 2                  | " "   |              | " "          |
| " 3                  | " "   |              | No illness   |
| " 4                  | " "   | No illness   | " "          |
| " 5                  | " "   | " "          | Died—80 hrs. |
| " 6                  | " "   | " "          | " 96 "       |
| " 7                  | " "   |              | " 90 "       |
| " 8                  | " "   | No illness   | No illness   |
| " 9                  | " "   | " "          | Died—96 hrs. |
| " 10                 | " "   | No illness   | " 96 "       |
|                      |   |              | No illness   |
|                      |   |              | Died—75 hrs. |
| Control sera         |   |              |              |
| 1444                 | Died—56 hrs.  |              |              |
| 1444                 | " 56 "  |              |              |
| 1444                 | " 61 "  |              |              |
| 1237                 |   | Died—66 hrs. |              |

These animals were thus considerably younger than those furnishing the hyper-immune anti-hog cholera serum or than those western hogs from which blood was obtained at slaughter. To test the possibility that the development of pseudorabies virus-neutralizing antibodies might be dependent upon the age of the animals rather than upon a previous attack of the disease, blood was obtained from 10 large hogs that had been born and reared on a local farm. These animals weighed from 250 to 350 pounds each and were approximately 1 year of age.

As shown in Table V, the serum of none of the large local hogs neutralized pseudorabies virus. This experiment indicates that the

age of the animals furnishing the serum was not of importance in determining the presence or absence of pseudorabies virus-neutralizing antibodies.

## *II. Rats in the Transmission of Pseudorabies among Swine*

In the preceding paper (1) brief mention was made of the fact that, among the earlier workers, both Schmiedhoffer (6) and Hutyra (7) and, more recently, Burggraaf and Lourens (8) suspected that rats

TABLE V

*The Absence of Pseudorabies Virus-Neutralizing Antibodies in the Blood Serum of Large Local Swine*

| Serum from swine No. | Amount of serum mixed with 100 mg. of<br>pseudorabies virus and administered<br>subcutaneously to guinea pigs |
|----------------------|---|
|                      | 1 cc.   |
|                      | Result  |
| T. J. 1              | Died—66 hrs.  |
| " " 2                | " 56 "  |
| " " 3                | " 64 "  |
| " " 4                | " 68 "  |
| " " 5                | " 68 "  |
| " " 6                | " 66 "  |
| " " 7                | " 73 "  |
| " " 8                | " 64 "  |
| " " 9                | " 80 "  |
| " " 10               | " 73 "  |
| Control sera         |   |
| 1444                 | Died—56 hrs.  |
| 1444                 | " 56 "  |

played some rôle in the transmission of pseudorabies to cattle. The exact manner in which rats could transmit the disease was never clearly envisaged. The repeated mention of the possibility in the literature and Hutyra's (7) direct evidence that the brains of rats, dead on a farm where pseudorabies was occurring in cattle, contained the specific virus made it seem possible that rats might play a rôle in the epidemiology of the disease. Could swine be infected with pseudorabies by

eating the carcasses of rats dead of the disease? Study of trichinosis has shown the great importance of rats in at least one ailment to which swine are susceptible. Here the accepted belief is that swine may become trichina-infected by eating infected rat carcasses; the rats may be kept infected by cannibalism among themselves or by eating the infected carcasses of other animals (9). Might not pseudorabies be spread from rats to swine in like manner? It was decided to test this possibility experimentally.

Earlier workers have claimed that rats could be infected with pseudorabies by feeding on virus-containing tissues of an animal dead of the disease (6, 10). Using wild brown rats (*Mus norvegicus*), this observation was confirmed.

Pseudorabies virus in the form of the brain of rabbits dead of the disease was mixed with corn-meal and placed in Petri dishes in the cages of recently captured wild brown rats. Although they ate but sparingly of the mixture, 2 out of the 9 animals thus fed developed pseudorabies and died. These showed the typical pseudorabies pruritus with self-mutilation localized to the lips and the skin about the mouth. Prolonged feeding of the virus-corn-meal mixture failed to infect the remaining 7 rats. Infections could, however, be quite regularly induced if the virus suspension was introduced directly into the mouth by pipette. Because of the great timidity of the wild rat in captivity, natural conditions as to feeding habits could not be imitated. It was a fact that the 2 rats that became infected by feeding on the virus-corn-meal mixture were the ones that ate most promptly. It is believed that the others may have failed to become infected because they delayed so long in eating their food that the virus became inactivated. White rats, accustomed to captivity, could be readily and regularly infected by feeding the virus corn-meal mixture. Both the white and the wild brown rats succumbed in from 3 to 7 days following feeding with pseudorabies virus.

The distribution of virus in a wild brown rat dead following feeding with virus by pipette was determined by rabbit inoculation. Virus was not present in demonstrable amounts in suspensions of liver and spleen. It was, however, present in the heart blood, lung, and brain.

From the preceding experiments it seemed established that wild brown rats could be infected with pseudorabies by feeding virus, that they developed a fatal disease, and that their carcasses contained virus. Experiments to determine whether swine could be infected by feeding on carcasses of wild rats dead of the disease were next conducted. Two swine were thus fed and both became infected. The animals ate the rat carcasses voraciously and with apparent relish. Swine 1562

was fed 2 rats on succeeding days but Swine 1539 was infected with equal facility by feeding only a single rat carcass. The rats used in

TABLE VI

*The Presence of Pseudorabies Virus in the Noses of Swine Infected by Feeding Carcasses of Wild Rats Dead of the Disease*

| Day of experiment | Swine 1562*               | Swine 1539†               | Swine 1551‡                   |
|-------------------|---------------------------|---------------------------|-------------------------------|
|                   | Fed carcass of Wild Rat 2 | Fed carcass of Wild Rat 1 |                               |
| 1                 | Fed carcass of Wild Rat 7 | 0                         |                               |
| 2                 | 0                         | 0                         |                               |
| 3                 | + (110 hrs.)              | X 0                       |                               |
| 4                 | + ( 85 " )                | X + (98 hrs.)             | Placed in pen with Swine 1539 |
| 5                 | + ( 76 " )                | X -                       | 0                             |
| 6                 | + ( 76 " )                | X -                       | 0                             |
| 7                 | X + ( 83 " )              | X + (92 hrs.)             | 0                             |
| 8                 | + (101 " )                | -                         | -                             |
| 9                 | + ( 88 " )                | + (87 hrs.)               | + ( 80 hrs.)                  |
| 10                | -                         |                           | X + (106 " )                  |
| 11                | -                         |                           | X + ( 77 " )                  |
| 12                | -                         |                           | + ( 87 " )                    |
| 13                | 0                         |                           | -                             |
| 14                | -                         |                           | K -                           |

X = temperature elevation to fever level (40°C. or higher).

0 = not tested for virus.

- = virus absent—test rabbit survived.

+ = virus present—test rabbit died of pseudorabies. (Hours in parentheses indicate time elapsing between inoculation and death.)

K = Swine 1551 was sacrificed—pseudorabies virus was present in nasal washings taken at autopsy. No virus demonstrable in brain, heart blood, spleen, liver, lung, salivary glands, dorsal cord, or Berkefeld filtered urine and feces suspension.

\* Nasal washings tested for virus by subcutaneous inoculation in rabbits.

† Presence of virus determined by rubbing swine's nose against scarified abdominal skin of rabbits.

these experiments had been infected with pseudorabies by feeding and were given to the swine very shortly after death.

Swine 1539 was placed in the same pen with a normal hog, Swine 1551, on the 4th day following feeding and infected this animal by

contact. Pseudorabies induced in swine by feeding was evidently communicable in the same manner as that following subcutaneous or intramuscular inoculation. Also, as shown in Table VI, virus was present in the noses of the infected swine.

The nasal washings of Swine 1562 contained virus for 7 successive days although the animal at no time appeared more than very mildly ill and had an elevation of temperature to fever level on only 1 day. The test for the presence of virus in the nose of Swine 1539 was conducted by rubbing it against the freshly scarified abdominal skin of rabbits. As pointed out in the preceding paper (1), this method probably gives less accurate information as to the presence or absence of virus in the nasal passages of infected swine than does that in which nasal lavage is employed. However, virus was demonstrable on the nose of Swine 1539 on each of 3 days and Swine 1551, placed in the same pen, developed pseudorabies by exposure. Tests for the presence of virus on the nose of Swine 1551, conducted in the same way as for Swine 1539, revealed its presence on 4 successive days.

These experiments suggest that rats may, theoretically at least, play a direct rôle in the epidemiology of pseudorabies in swine and thus an indirect rôle in the bovine disease. They furnish a possible explanation of means by which the disease may spread from one swine herd to the next, other than by direct contact. Because of the highly contagious nature of pseudorabies in swine, it is necessary only to assume that a single hog become infected with the disease by eating the carcass of an infected rat in order to establish pseudorabies in the entire swine herd, thus setting up a potential source of infection for any associated cattle.

#### DISCUSSION

The results of experiments designed to gain information concerning the occurrence of pseudorabies among middle western swine have suggested that it is a very prevalent disease. Using the presence of specific virus-neutralizing antibodies in the serum as a criterion indicative of an earlier infection with the virus, pooled samples of serum from large numbers of swine have been studied. In only 2 out of 23 such serum samples was it not possible to demonstrate that at least one of the animals contributing to the sample had had an earlier



pseudorabies infection. In the remaining 21 samples, the virus-neutralizing titer was such as to indicate that from 5 per cent to 50 per cent of the animals had recovered from an earlier pseudorabies. In like manner, 13 out of 15 swine of middle western origin bled at the laboratory of a biological house, and 10 middle western swine picked at random from the killing platform of a local slaughterhouse were found to be pseudorabies-immune as judged by the presence of specific virus-neutralizing antibodies in their sera. In contrast to the relatively large numbers of middle western swine whose sera have been found to contain pseudorabies virus-neutralizing antibodies may be mentioned the complete absence of similar neutralizing antibodies in the sera of swine raised on our own farm and on other farms in this section of New Jersey.

The data furnished by the serum samples that were studied may not accurately represent the incidence of pseudorabies among swine on middle western farms. The hogs supplying these sera had in all probability been shipped and reshipped and passed through a series of stockyards between the time they left the farm and the time they were bled either at the laboratories of biological houses or at a slaughterhouse. A question which naturally arises and which cannot at present be answered is whether these animals acquired their pseudorabies infection while still on their home farms, at some time during their travels, or even in the yards where they were quartered while being hyperimmunized against hog cholera. The fact that the incidence of infection, as judged by the virus-neutralizing titer of the sera, did not, in a number of instances, approach 100 per cent would suggest that the infections had been suffered before the swine reached their final destinations. If they occurred before the animals left the farms, then the data given in Tables II, III, and IV suggest that the disease is really prevalent in the middle West to a startling extent. If they occurred after the animals left the farms then it must be concluded that the chances of swine acquiring pseudorabies during shipping or passage through stockyards are indeed very great.

A possibility was entertained for a while that the hog cholera virus used in vaccinating swine might be contaminated with pseudorabies virus and might thus be responsible for the dissemination of the disease. However, hog cholera virus obtained from 9 different biological houses

was found to be free of pseudorabies virus, as tested by rabbit inoculation, effectively eliminating this possibility from consideration.

In view of the apparently high incidence of pseudorabies among middle western swine and situation of the virus in and on the pigs' noses, it is reasonable to suppose that it might occasionally spread to cattle. Indeed, when the almost universal practice of keeping cattle and swine in the same enclosures on middle western farms is considered, it is surprising that cattle are not often infected. A possible explanation of the relatively low incidence may be that swine acquire their infection when quite young and before they reach a size or age at which they begin "following" cattle. Under such conditions the disease might be expected to be relatively uncommon in cattle and only transmitted to them by hogs that escaped infection until they were mature enough to come into intimate contact with cattle.

Swine influenza is the only clinically recognized disease of hogs in the middle West that has an incidence of infection even approaching that suggested by the present data for pseudorabies. This disease reaches epizootic proportions in many autumns and even in the inter-epizootic years it is quite prevalent. Like pseudorabies, influenza in swine is highly contagious and its morbidity rate in animals under 1 year of age in an infected herd approaches 100 per cent. Köves and Hirt (5) were greatly impressed by the similarity between pseudorabies in swine as observed by them in Hungary and descriptions of swine influenza as occurring in America. They have suggested that the two diseases may be etiologically related. However, much as the two maladies may resemble each other, their etiological viruses can be shown to be entirely dissimilar. Pseudorabies virus regularly produces fatal infections when administered subcutaneously to any of the

ment, and in cattle and the small laboratory animals one of the most regularly fatal diseases known. Superficially it would appear that the state of parasitism between virus and host is more perfect in the case of swine than in the cases of other susceptible animals. Whether this has been achieved through long years of infection of swine with the virus, with a consequent gradual adaptation of parasite to host, or whether some physiological peculiarity of swine is responsible, cannot be determined from present knowledge of the disease. It seems certain, however, that, were it not for the occasional transmission of pseudorabies to cattle, its existence in swine in this country would not have been suspected.

The observation that wild brown rats can be infected with pseudorabies by feeding, and that their carcasses, in turn, give rise to the disease when fed to swine introduces the interesting possibility that they may play an epidemiological rôle in the spread of the disease to cattle. Although suspected by earlier workers of being in some way responsible for outbreaks of pseudorabies, the exact manner in which rats transmitted their infection was never explained. The present experiments suggest that their epidemiological rôle in bovine pseudorabies is probably only an indirect one and that swine are the intermediate host through which this indirect transmission of the virus from rats to cattle is effected.

The final epidemiological set-up may be visualized approximately as follows:—

Pseudorabies infection among wild rats may be maintained in much the same fashion as trichina infection, by cannibalism or by the ingestion of virus-containing tissues from other animals. The disease may be transmitted to swine when the carcass of a pseudorabies-infected rat is eaten by a hog. From this hog the disease spreads to other swine in the same herd as a contagious disease. It is transmitted to swine herds on other farms, either by direct contact of infected swine with normal swine or by the migration of infected rats. With two such efficient modes of dissemination of the virus among swine, one would expect the disease to be prevalent in this species. However, because of the extremely mild nature of porcine pseudorabies its existence is not suspected. Only when the virus breaks away from this swine reservoir and spreads to cattle is its presence on mid-western farms made known. The transmission of the virus from swine to cattle, as

pointed out earlier (1) is thought to take place when the noses of infected hogs come in contact with abraded areas of skin on cattle. Carcasses of cattle dead of the disease, if gnawed by rats, serve as a fresh source of virus from which a rat population can become infected. A cycle from rat, to swine, to cow, and back to rat can thus be completed.

The experiments presented in this and the preceding paper (1) furnish an explanation of the means by which pseudorabies is transmitted to cattle under natural conditions. They are not advanced as proving that the infection actually takes place in the manner suggested but rather that it may take place in this way. Only further carefully controlled field experiments can determine whether the widespread, unrecognized pseudorabies infection in swine serves as the reservoir from which cattle are directly infected.

#### SUMMARY

Study of the pseudorabies virus-neutralizing antibody content of pooled and individual samples of swine serum have led to the conclusion that pseudorabies is a highly prevalent, unrecognized, disease in middle western hogs.

It has been shown that wild brown rats develop a fatal infection following ingestion of pseudorabies virus and that their carcasses, in turn, give rise to the disease in swine to which they are fed. It is believed that rats play a rôle in the epidemiology of porcine pseudorabies, serving as the initial source of infection for a swine herd and also as one means by which virus can be spread from farm to farm in swine herds. The experiments presented furnish further evidence that swine may serve as the source of infection for cattle.

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# EFFECT OF ANAEROBICALLY PREPARED PNEUMOCOCCUS AUTOLYSATE TOXIN ON MICE AND EVALUATION OF PNEUMOCOCCUS AUTOLYSATE ANTITOXIN IN MICE

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In previous papers (1, 2) we showed that the Berkefeld filtrates of certain anaerobically produced autolysates of pneumococcus of Types I, II, or III cause in guinea pigs: (a) necrosis of the skin when injected intracutaneously; (b) hemorrhagic edema of the lungs and death when injected intratracheally; and (c) pneumonia associated with unrestrained multiplication of the organism in the lung when injected intratracheally in sublethal doses combined with living pneumococci. The absorption of this autolysate poison with red blood cells removed the factor injurious to the lung but left the power to produce necrosis of the skin unimpaired, showing that at least 2 toxic substances were present in the autolysates (3). We also demonstrated that immunization of rabbits and especially of horses with these autolysates stimulates the production of sera of marked antitoxic properties both for the principle causing skin necrosis and the principle causing hemorrhagic edema of the lungs (1, 4, 5). Moreover, an anti-autolysate serum prepared by the immunization with the autolysate from one type of pneumococcus neutralized the poisons affecting the skin and the lung produced from each of the other three types, which demonstrated that these antitoxins, and presumably the toxins, were not type specific (1, 4, 5).

In a series of papers in 1931 and 1934, Blackman and his associates have shown that our anaerobically prepared pneumococcus autolysate poison when injected intravenously into rabbits produce septicemia or sepsis, depending on the dosage of the toxin and the immunity of the animal to the poison (6, 7).

Recently we found that these pneumococcus autolysates are also toxic for mice when injected intravenously in comparatively small doses and that their toxic effects may be neutralized by the antitoxin to the autolysate in relatively high dilutions. Since the intravenous method of injection in mice for the evaluation of pneumococcus autolysate antitoxin has many advantages, such as simplicity, economy, accuracy, over the previous intratracheal method in guinea pigs, this study was undertaken. In addition to giving the details of the titration of the pneumococcus autolysate antitoxin in mice when this antitoxin and autolysate toxin are mixed before injection, we shall show that this antitoxin is also efficacious when given separately either at the same time as or 2 hours after the injection of the poison.

### *Methods*

The preparation of the toxic autolysates has been described in detail in previous papers. Briefly, the method is to autolyze in broth at room temperature under vaseline seal, the centrifuged pneumococci obtained after 18 hours growth in double strength veal infusion broth containing 4 per cent Witte peptone. No phenol was added to any of these preparations. In the experiments here reported all autolysate poisons were prepared from a Type II pneumococcus recovered from a case of lobar pneumonia at the Presbyterian Hospital. The pneumococcus strain was kept virulent by passage through rabbits.

*Toxicity of the Autolysates for Mice.*—The autolysate toxin injected intravenously in 0.1 to 0.2 cc. quantities kills 18–20 gm. mice in a few minutes to 8 days depending on the strength of the toxin and the usual individual differences in the susceptibility of the mice. In the mice that die quickly, within 2 hours, the symptoms develop soon after the injection and consist of weakness and increasing prostration until death. The mice that survive 2 or 3 days have the same symptoms as those described above, preceded by an incubation period of 2 or 3 hours in which they appear normal. Sometimes convulsions occur in these latter animals before death.

At autopsy, in the mice that die within a few hours, nothing abnormal is seen in the gross, except tiny hemorrhages in the lungs. In those that survive 2 to 4 days, usually the organs appear normal in the gross except for the kidneys which are enlarged and of a pale color. In these latter mice and in those that survive longer than 4 days, marked edema of the subcutaneous tissues or ascites or both edema and ascites may occur, lasting for variable periods. The kidneys of the mice that survive 5 to 8 days are usually markedly enlarged, 2 to 3 times their normal size, and of a white or pale yellow color. Many of the mice having the large white or pale yellow kidneys are much emaciated at the time of death. Most of these latter mice lose one-third their weight and some more than one-half their

weight after injection. In these emaciated mice the tissues are pale and, except for the kidneys that, as stated before, are remarkably enlarged, the organs and tissues appear shrunken. Massive albuminuria appearing 18 hours after injection and continuing for 4 or 5 days was found.

The pathology of mice inoculated with the pneumococcus autolysate poison will be taken up in detail in a later publication.

*Autolysate Antitoxic Sera.*—Autolysate antitoxins 1, 2, and 3—concentrated and refined sera obtained from horses immunized for us by Eli Lilly and Company in 1928 and 1929 with the sterile toxic pneumococcus Types I, II, and III.

Autolysate antitoxin 4—concentrated and refined serum obtained from horses immunized by Eli Lilly and Company in 1928 and 1929 with the sterile toxic autolysate filtrates from Pneumococcus Types I, II, and III combined with dead pneumococcus organisms, Types I, II, III, and IV. Autolysate antitoxins 1, 2, 3, and 4 are the same that we titrated previously in 1928 and 1929 for antitoxins to the toxic effect for the lung. Recent tests by the intratracheal method in guinea pigs demonstrate that these antitoxins have lost in the last 5 years over one-half their neutralizing antibodies for the lung poison.

Autolysate antitoxin 5—preparation 2 re-refined in August, 1934, by Eli Lilly and Company.

*Antibacterial Sera.*<sup>1</sup>—Antibacterial serum 6—New York Board of Health, Types I and II, refined, containing 1500 units per cc. of Type I and 800 units per cc. of Type II.

Antibacterial serum 7—New York Board of Health, Type I, refined, containing 2500 units per cc. of Type I.

Antibacterial serum 8—New York Board of Health, Types II and V, refined, containing 800 units per cc. of Type II and 1500 units per cc. of Type V.

Normal horse serum, inactivated.

#### *Evaluation of Pneumococcus Autolysate Antitoxin When the Autolysate Toxin and Serum Are Incubated Together before Infection*

In this work 1 unit of toxin is taken as the smallest amount of pneumococcus toxic autolysate that kills 18–20 gm. mice regularly in less than 2 days when inoculated intravenously; and 1 unit of antitoxin represents the smallest amount of serum that, when incubated at 37°C. for 30 minutes with 1 unit of toxin, neutralizes the toxin so that no symptoms occur when it is injected intravenously into mice.

<sup>1</sup>The pneumococcus antibacterial sera were obtained from Dr. Jesse B. G. and Miss Georgia Cooper.



The tests for the presence of neutralizing antibodies were carried out as follows:

Dilutions of the sera in physiological salt solution were first prepared. 0.1 cc. of the various serum dilutions or salt solution instead of the serum dilutions were pipetted into separate precipitin tubes. To each tube 0.9 cc. of toxic pneumococcus autolysate, if necessary, diluted with saline so that it contained 10 units, was added and the contents of each tube immediately mixed. The tubes were placed in the water bath at 37°C. for 30 minutes and then in ice water until used. 0.2 cc. from each tube, representing 2 units of toxin, was inoculated intravenously into one or more mice weighing 18–20 gm.

Normal horse serum and specific antibacterial pneumococcus sera were used to control the pneumococcus autolysate antitoxins.

A typical experiment demonstrating the neutralizing action of several pneumococcus autolysate antitoxins on one toxic autolysate is presented in Table I. Because of lack of sufficient autolysate toxin the antitoxins were not titrated to their end-points. However, it is seen that autolysate antitoxins 1, 2, and 3, contain at least 200 mouse units per cc., whereas the antibacterial serum 6, containing 1500 and 800 protective units for *Pneumococcus* Types I and II, respectively, even in 1–10 dilution has no neutralizing properties for our autolysate toxin.

In Table II, using a different autolysate toxin, it is seen that 3 antibacterial sera in 1–10 dilutions and normal horse serum in 1–20 or 1–50 dilutions have slight if any detoxicating effect on this poison. However, horse serum when incubated with this toxin in 1–10 dilution does appear to have resulted in the delayed death of 2 mice injected with such mixtures. (See Table II, Mice 1 and 2.)

*The Protection of Mice against the Lethal Action of Pneumococcus Autolysate Toxin by the Separate Treatment with Pneumococcus Autolysate Antitoxin*

Table III gives the results obtained in an experiment in which autolysate antitoxic serum was given intraperitoneally immediately after the intravenous injection of a lethal dose of autolysate toxin, normal horse serum and antibacterial serum being used as controls. All the sera were diluted 1–5 with salt solution and 0.5 cc. of these dilutions was given in each case as indicated in the table. The poison

used in this experiment was a very weak preparation. Table III is self-explanatory.

In Table IV is presented an experiment in which the pneumococcus autolysate antitoxin and 2 antibacterial sera as controls were given

TABLE I  
*Titration of Pneumococcus Autolysate Antitoxin*

| Serum                    | Serum dilutions                         |          |          |           |          |                                |
|--------------------------|---|----------|----------|-----------|----------|--------------------------------|
|                          | 1-10                                    | 1-20     | 1-50     | 1-100     | 1-200    | 1-400                          |
| Autolysate anti-toxin 2  | —                                       | —        | —        | S         | S        | D 18 hrs.                      |
| Autolysate anti-toxin 1  | —                                       | —        | —        | S<br>S    | S<br>S   | D 3 days<br>D 3 "<br>D 18 hrs. |
| Autolysate anti-toxin 3  | —                                       | —        | —        | S         | D 3 days | —                              |
| Autolysate anti-toxin 4  | —                                       | —        | —        | S         | S        | —                              |
| Autolysate anti-toxin 5  | —                                       | —        | —        | D 4 days  | D 1 day  | —                              |
| Antibacterial serum 6    | D 2 hrs.<br>D 5 "                       | D 6 hrs. | D 6 hrs. | D 20 min. |          |                                |
| Control toxin and saline | D 2 min.<br>D 5 "<br>D 40 "<br>D 6 hrs. | —        | —        | —         | —        | —                              |

mice injected with autolysate antitoxin survived, whereas all the control mice died. The mouse that died after antitoxin treatment was already extremely sick when injected with antitoxin. In this experiment, Table IV, it is seen that there is great variation in the time of

TABLE II

*The Effect of Normal Horse Serum and 3 Antibacterial Pneumococcus Sera When Incubated with Pneumococcus Autolysate Toxin before Injection*

| Mouse No. | Material inoculated  | Final dilution of serum | Dead    |
|-----------|--|-------------------------|---------|
| 1         | Pneumococcus autolysate toxin and normal horse serum                         | 1-10                    | 5 days  |
| 2         | " "  | 1-10                    | 5 "     |
| 3         | " "  | 1-20                    | 2 "     |
| 4         | " "  | 1-20                    | 18 hrs. |
| 5         | " "  | 1-50                    | 2 days  |
| 6         | " "  | 1-50                    | 2 "     |
| 7         | Pneumococcus autolysate toxin and antibacterial serum Type I (No. 7)         | 1-10                    | 18 hrs. |
| 8         | " "  | 1-10                    | 4 days  |
| 9         | " "  | 1-10                    | 4 "     |
| 10        | Pneumococcus autolysate toxin and antibacterial serum Types I and II (No. 6) | 1-10                    | 4 "     |
| 11        | " "  | 1-10                    | 18 hrs. |
| 12        | " "  | 1-10                    | 4 "     |
| 13        | Pneumococcus autolysate toxin and antibacterial serum Types II and V (No. 8) | 1-10                    | 4 days  |
| 14        | " "  | 1-10                    | 2 "     |
| 15        | " "  | 1-10                    | 4 "     |
| 16        | Pneumococcus autolysate toxin and saline                                     | —                       | 3 "     |
| 17        | " "  | —                       | 30 min. |
| 18        | " "  | —                       | 2 days  |
| 19        | " "  | —                       | 18 hrs. |

0.9 cc. of autolysate toxin and 0.1 cc. of serum, serum dilution, or saline. In water bath at 37° for 30 minutes. Mice inoculated intravenously with 0.2 cc. of mixtures.

death in the 5 mice inoculated with the antibacterial Type I and II serum (No. 6). That mice inoculated intravenously with this serum 2 hours after the injection of autolysate poison often die sooner than the other controls, has been our experience in other experiments. No explanation of this observation is apparent at present. The serum in

TABLE III  
*The Effect of Pneumococcus Autolysate Antitoxin Given Intraperitoneally Immediately after the Intravenous Inoculation of a Lethal Injection of Pneumococcus Toxin*

| Mouse No. | Pneumococcus autolysate toxin cc. | Serum intraperitoneally 0.5 cc. of 1-5 dilution |                  |
|-----------|-----------------------------------|---|------------------|
|           |                                   |   | Died or survived |
| 1         | 0.2                               | Normal horse                                    | D 3 days         |
| 2         | 0.2                               |   |                  |
| 3         | 0.2                               | Antibacterial serum Types I and II (No. 6)      | D 2 "            |
| 4         | 0.2                               |   |                  |
| 5         | 0.2                               | Autolysate antitoxin 1                          | D 2 "            |
| 6         | 0.2                               |   |                  |
| 7         | 0.2                               | —   | Survived         |
| 8         | 0.2                               |   |                  |
|           |                                   | —   | D 4 hrs.         |
|           |                                   | —   | D 4 days         |
|           |                                   |   | D 3 "            |

TABLE IV  
*The Effect of Pneumococcus Autolysate Antitoxin Given Intravenously 2 Hours after the Intravenous Inoculation of a Lethal Dose of Pneumococcus Autolysate Toxin*

| Mouse No. | Pneumococcus autolysate toxin cc. | Serum intravenously 0.5 cc. of 1-5 dilution |                  |
|-----------|-----------------------------------|---|------------------|
|           |                                   |   | Died or survived |
| 1         | 0.2                               | Autolysate antitoxin 1                      | D 20 min.        |
| 2         | 0.2                               |   |                  |
| 3         | 0.2                               |   |                  |
| 4         | 0.2                               |   |                  |
| 5         | 0.2                               |   |                  |
| 6         | 0.2                               |   |                  |
| 7         | 0.2                               | Antibacterial serum Type I (No. 7)          | S                |
| 8         | 0.2                               |   |                  |
| 9         | 0.2                               |   |                  |
| 10        | 0.2                               |   |                  |
| 11        | 0.2                               |   |                  |
| 12        | 0.2                               |   |                  |
| 13        | 0.2                               | Antibacterial serum Types I and II (No. 6)  | D 54 hrs.        |
| 14        | 0.2                               |   |                  |
| 15        | 0.2                               |   |                  |
| 16        | 0.2                               |   |                  |
| 17        | 0.2                               |   |                  |
| 18        | 0.2                               |   |                  |
| 19        | 0.2                               | —   | D 1 hr.          |
| 20        | 0.2                               |   |                  |
| 21        | 0.2                               |   |                  |
| 22        | 0.2                               |   |                  |
| 23        | 0.2                               |   |                  |
| 24        | 0.2                               |   |                  |
| 25        | 0.2                               | —   | D 30 hrs.        |
| 26        | 0.2                               |   |                  |
| 27        | 0.2                               |   |                  |
| 28        | 0.2                               |   |                  |
| 29        | 0.2                               |   |                  |
| 30        | 0.2                               |   |                  |
| 31        | 0.2                               | —   | D 15 "           |
| 32        | 0.2                               |   |                  |
| 33        | 0.2                               |   |                  |
| 34        | 0.2                               |   |                  |
| 35        | 0.2                               |   |                  |
| 36        | 0.2                               |   |                  |
| 37        | 0.2                               | —   | D 1 hr.          |
| 38        | 0.2                               |   |                  |
| 39        | 0.2                               |   |                  |
| 40        | 0.2                               |   |                  |
| 41        | 0.2                               |   |                  |
| 42        | 0.2                               |   |                  |
| 43        | 0.2                               | —   | D 3 hrs.         |
| 44        | 0.2                               |   |                  |
| 45        | 0.2                               |   |                  |
| 46        | 0.2                               |   |                  |
| 47        | 0.2                               |   |                  |
| 48        | 0.2                               |   |                  |
| 49        | 0.2                               | —   | D 5 hrs.         |
| 50        | 0.2                               |   |                  |
| 51        | 0.2                               |   |                  |
| 52        | 0.2                               |   |                  |
| 53        | 0.2                               |   |                  |
| 54        | 0.2                               |   |                  |
| 55        | 0.2                               | —   | D 4 "            |
| 56        | 0.2                               |   |                  |
| 57        | 0.2                               |   |                  |
| 58        | 0.2                               |   |                  |
| 59        | 0.2                               |   |                  |
| 60        | 0.2                               |   |                  |

the quantity used is not toxic in itself for mice. On the other hand, the delayed death of Mouse 12 in this experiment appears to be due to an individual resistance of this mouse to the poison.

When mice are injected with autolysate toxin mixed with adequate (neutralizing) amounts of autolysate antitoxin or separately with toxin and adequate amounts of antitoxin and are killed at varying intervals after such injections, no edema, emaciation, or macroscopic kidney changes are seen at autopsy. Neither is there albumin in the urine of these animals. On the other hand, when mice are inoculated with partly neutralized mixtures of autolysate toxin and autolysate antitoxin or with toxin followed by inadequate amounts of antitoxin, prolonged intoxication results with death in 5 to 8 days and the usual findings of animals dying slowly from injection of pneumococcus autolysate toxin alone; *i.e.* emaciation, large white or pale yellow kidneys, albuminuria, and occasional edema.

#### DISCUSSION

The experiments reported in this paper appear to demonstrate that certain anaerobically prepared autolysates of pneumococcus cause death when injected intravenously into mice and that antitoxin produced in horses by immunization with the toxic autolysates neutralizes the poison so that no toxic effects result from its inoculation. At present we have no conclusive evidence as to whether or not the substance or substances in the autolysate toxic for mice are identical with the substance or substances injurious to the lungs of guinea pigs. In favor of the view that the toxic effects in mice and in guinea pigs are due to the same poisonous principle are the findings (not reported in this paper) that heating the toxic autolysates to 60°C. for 10 minutes destroys both toxic effects, and that the substance toxic for mice is absorbed from toxic autolysate filtrates by red cells and red cell stroma as is the substance injurious to the lungs of guinea pigs.

In previous papers it was established that the principle in the autolysates injurious to lungs is antigenically similar for *Pneumococcus* Types I, II, and III. Unfortunately at the time of carrying on the experiments reported in this paper no pneumococcus autolysate monovalent antitoxic serum was available, which fact prevented our determining whether the poisonous principle for mice in the toxic autolysates is also species specific for *Pneumococcus* Types I, II, and III

and not type specific. However, we did test autolysate antitoxin 1 against a toxic autolysate prepared from *Pneumococcus* Type 19 strain<sup>2</sup> and found that it neutralized this autolysate toxin similarly to the *Pneumococcus* Type II autolysate toxin. Therefore, as far as these experiments go, the evidence is that the substance in the autolysates that kills mice is species specific and not type specific.

#### CONCLUSIONS

1. Certain anaerobically produced autolysates of *pneumococcus* injected intravenously kill mice in 0.1 to 0.2 cc. quantities in a few hours to 8 days.
2. The symptoms of mice inoculated with these autolysates are weakness and increasing prostration until death. Massive albuminuria is found, appearing 18 hours after injection. During the course of a prolonged intoxication ascites and edema of the subcutaneous tissues may develop. Large pale yellow or white kidneys are found in mice that survive 5 days. In these latter animals, emaciation is usually marked at death.
3. The antitoxin prepared in horses by immunization with the anaerobically prepared toxic autolysates of *pneumococcus* neutralizes the poisonous autolysates whether mixed *in vitro* before injection or injected separately after the autolysate.
4. The injection of *pneumococcus* autolysate toxin incompletely neutralized with autolysate antitoxin causes a protracted intoxication with symptoms and pathologic findings similar to those found in mice dying slowly after injections of the toxic autolysate alone.

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- <sup>1</sup>The *Pneumococcus* Type 19 strain was obtained from Dr. Harriet Alexander of the Johns Hopkins.



# STUDIES ON THE IMMUNE RESPONSE OF THE RHEUMATIC SUBJECT AND ITS RELATIONSHIP TO ACTIVITY OF THE RHEUMATIC PROCESS\*

## I. THE DETERMINATION OF ANTISTREPTOLYSIN TITER

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Todd (1) first demonstrated that hemolytic filtrates from streptococcal cultures grown in serum-free broth are antigenic and later (2) showed that serum streptolysin is also an antigen. Both types of streptolysin were found to be equally antigenic. The antibody produced by inoculating either of these antigens into animals neutralized serum-free streptolysin but failed to neutralize serum streptolysin. Further observations (2) indicated that the streptolysin found *in vivo* is probably more closely related to serum streptolysin than to serum-free streptolysin. Examination of sera from normal horses revealed the presence of antistreptolysin in extremely small quantity. High antistreptolysin titers were found in sera of those horses which had been immunized with *Streptococcus hemolyticus*. Furthermore, a rise in the antistreptolysin titer was demonstrated in the sera of human subjects convalescing from respiratory infection with hemolytic streptococcus; while individuals recovering from diseases with other infectious agents failed to develop this antibody (3).<sup>1</sup> The presence of a high antistreptolysin titer in the sera of patients experiencing an acute attack of rheumatic fever, in conjunction with other supportive findings, was considered by the present authors (4) to be a link in the

\* The work reported in this communication was carried out under The Kellogg Foundation Fund.

<sup>1</sup> Further observations made in this laboratory on rheumatic patients convalescent from lobar pneumonia, Pneumococcus Types I and II, and on those patients recovering from a variety of infections, have been in accord with the previous observations of Todd.



chain of evidence leading to the hypothesis that the rheumatic process may be activated by respiratory infection with *Streptococcus hemolyticus*.

The purpose of this series of papers is to direct attention to the close relationship which exists between the development of the immune response to this infection and initiation of rheumatic activity in susceptible subjects. It includes clinical, bacteriological and immunological observations which have been collected over a period of years. It concerns itself with an interpretation of more than 3,000 antistreptolysin determinations made on approximately 500 human subjects including infants, children and adults to the age of fifty. This paper deals first with the method used throughout the entire study; and second with the natural level of antistreptolysin in human subjects, as a basis of reference for the papers to follow.

### A. Technique

Since the original findings of Todd (1) certain refinements in technique have been devised by him (5); also by Hodge and Swift (6, 7) and by the authors. These have been employed in all the determinations listed in the present report. The technique has been found to be accurate, and the antistreptolysin titers of a standard horse serum and of a human serum, kept under sterile conditions at 4°C., have remained constant during a period of 3 years. A detailed description of the methods used follows.

1. *Preparation of Streptolysin Broth*.—10 pounds of fresh beef hearts freed of fat and sinew are finely minced and infused overnight in a refrigerator with 10 liters of distilled water.

The next morning, with thorough and frequent stirring, the infusion is slowly heated over a free flame to 85°C. and maintained at that temperature for 30 minutes. The infusion is next squeezed through a wire mesh strainer and then passed through ordinary filter paper. Difco proteose peptone, 200 gm., is shaken lightly over the surface of the warm infusion, dissolving in 10 to 15 minutes *without further heating*. During the process of peptone solution, the following c.p. ingredients are added: dextrose 20 gm.; NaHCO<sub>3</sub> 20 gm.; Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 10 gm.; NaCl 20 gm. The reaction is adjusted to pH 8 with normal NaOH, after which the broth is refrigerated overnight.

The following morning, after filtering through standard filter paper, the medium is sterilized by passing through a battery of six Pasteur-Chamberland F candles, 200 by 25 mm., directly into 2,000 cc. pyrex calibrated filtering flasks. The broth is then incubated for 48 hours at 37°C. as a test for sterility. It is stored in the refrigerator at 4°C.

2. *Preparation of Streptolysin Using Todd's Strain Aronson (Schnitzer)*.—Seed

cultures consisting of 5 cc. of warm phosphate infusion broth containing 0.3 per cent dextrose are inoculated with one loop of stock culture of Aronson (Schnitzer) strain and shaken hourly during 5 or 6 hours incubation. As a precaution, the growth is then examined microscopically and blood agar plates are prepared. If microscopic examination shows pure culture of streptococcus, the large flasks of broth are then inoculated.

To each flask (1,500 cc.) of warmed broth are added 3 to 4 cc. of seed culture. The flasks are incubated 14 or 15 hours (*not* longer), smeared, plated and immediately chilled in cold running water, followed by refrigeration to prevent further growth and autolysis of organisms. The purity having been ascertained, the broth culture is next centrifuged and the supernatant fluid is filtered through a battery of Pasteur-Chamberland F candles *directly* into 2,000 cc. pyrex filtering flasks and stored in the refrigerator at 4°C.

3. (a) *Reduction of Lysin Broth*.—The lysin (any amount) is reduced by removal of oxygen under vacuum, 29 inches for 5 hours, with the lysin packed in ice, and by adding 1 per cent normal NaOH and 0.1 per cent finely powdered sodium hydrosulfite at a time convenient to the worker.

(b) *Storage of Reduced Lysin*.—Warm sterile vaseline is layered over the lysin to a depth of 2 cm., formation of bubbles being avoided. Tubes or 50 cc. Florence flasks may be conveniently employed. It has been found in this laboratory that a stable lysin maintains a constant titer over a period of months at 4°C. under these conditions of reduction.

4. *Titration of Lysin*.—At least 2 weeks are allowed to pass after reduction before the first titrations of new lysins are done. Samples from each flask, which has been reduced, are tested to determine the standard combining power according to the method of Hodge and Swift (7). These workers have demonstrated that although the titer of streptolysin varies, "its power of combining with antistreptolysin is constant under certain conditions." The standard serum used in this laboratory is an antistreptolysin globulin prepared by Dr. E. W. Todd. After their combining unit has been established, the lysins are standardized against human sera of known titer, high, medium and low units. The standardization is repeated four or five times with different rabbit cells in order to confirm the original combining power. The "combining unit" is, as described by Hodge and Swift, the largest quantity of streptolysin which causes no hemolysis in the presence of a standard amount of antistreptolysin. This is determined by titrating dilutions of streptolysin of unknown strength, against a constant volume of standard serum diluted with saline so that 1 cc. of the serum mixture contains 1:20,000 dilution of Todd's standard globulin (1.0 cc. of which contains 20,000 antistreptolysin units). The final percent of tubercles is given below.

|                                     | Volumes |      |     |      |     |      |     |      |     |  |  |
|-------------------------------------|---------|------|-----|------|-----|------|-----|------|-----|--|--|
|                                     | cc.     | cc.  | cc. | cc.  | cc. | cc.  | cc. | cc.  | cc. |  |  |
| Streptolysin.....                   | 0.5     | 0.45 | 0.4 | 0.35 | 0.3 | 0.25 | 0.2 | 0.15 | 0.1 | Incubated for 15 min. in<br>37°C. water bath be-<br>fore addition of cells |  |
| Saline.....                         | —       | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.35 | 0.4 |  |  |
| Standard globulin<br>1:200,000..... | 1.0     | 1.0  | 1.0 | 1.0  | 1.0 | 1.0  | 1.0 | 1.0  | 1.0 |  |  |
| 5% washed rabbit<br>cells.....      | 0.5     | 0.5  | 0.5 | 0.5  | 0.5 | 0.5  | 0.5 | 0.5  | 0.5 |  |  |

The tubes are incubated 15 minutes, then individually shaken, reincubated for 45 minutes and read.

By the use of this method it is possible to maintain a constant combining unit, providing the lysin has been kept thoroughly sealed. This method is more satisfactory than the one which uses 2.5 M.H.D. (Todd's original technique) which may vary with the character of the red cells of rabbits.

5. (a) *Determination of Titer of Antistreptolysin in Human Serum.* The *Antistreptolysin Test*.—All sera, irrespective of age, are inactivated for 30 minutes at 56°C. to prevent hemolysis by natural cytolysin in sera of low antistreptolysin titer.

In order to determine the range of hemolysis a preliminary titration of serum is made using 1 in 10, 1 in 50, 1 in 100 and possibly 1 in 500 dilutions. To each 1 cc. of serum dilution is added one combining unit of lysin diluted to 0.5 cc. It is essential that each tube be individually and thoroughly shaken after the addition of the lysin. This applies throughout all titrations after each addition. After thorough mixing, the tubes are incubated for 15 minutes in a water bath at 37°C. Then 0.5 cc. of 5 per cent washed rabbit red blood cells is added to each tube, which is shaken immediately and thoroughly. The mixture is incubated for 15 minutes, and the tubes are shaken again. After this they are reincubated, and the readings are made 45 minutes later. The tube which then shows no hemolysis contains the neutralizing dose and indicates the dilution of serum to be used in the final titration.

(b) *Final Titration*.—The final titration is made as follows: A series of tubes is set up, containing from 1 cc. to 0.1 cc. of the neutralizing serum dilution previously determined. To each of these is added one combining unit of streptolysin made up to 0.5 cc. in saline. All tests are always carefully controlled by comparison with the standard horse serum and human sera of known high, medium and low titers. The hemolytic activity of the lysin is also controlled in each test. The neutralizing dose of antistreptolysin in human sera has been found to range between 0.1 cc. and 0.0002 cc. of undiluted serum corresponding to the dilutions shown in Table I. Throughout this study the values are expressed in units per cubic centimeter, which is the reciprocal of the N.D., in order to facilitate com-

parison with the findings of other workers. In later papers of the present series, the authors have also used the logarithms of the units ( $\log u$ ), for the purpose of

TABLE I  
*The Neutralizing Dose of Antistreptolysin Corresponding to Various Serum Dilutions*

| Serum dilution | Cc. | Serum dilution | Cc. | Serum dilution | Cc. | Serum dilution | Cc. | N.D. of undiluted serum | Units per cc. |
|----------------|-----|----------------|-----|----------------|-----|----------------|-----|-------------------------|---------------|
| 1:10           | 1.0 |                |     |                |     |                |     | cc.                     |               |
|                | 0.9 |                |     |                |     |                |     | 0.1                     | 10.0          |
|                | 0.8 |                |     |                |     |                |     | 0.09                    | 11.1          |
|                | 0.7 |                |     |                |     |                |     | 0.08                    | 12.5          |
|                | 0.6 |                |     |                |     |                |     | 0.07                    | 14.2          |
|                | 0.5 |                |     |                |     |                |     | 0.06                    | 16.7          |
|                | 0.4 |                |     |                |     |                |     | 0.05                    | 20.0          |
|                | 0.3 |                |     |                |     |                |     | 0.04                    | 25.0          |
|                | 0.2 |                |     |                |     |                |     | 0.03                    | 33.3          |
|                |     | 1:50           | 1.0 |                |     |                |     | 0.02                    | 50.0          |
|                |     |                | 0.9 |                |     |                |     | 0.018                   | 55.6          |
|                |     |                | 0.8 |                |     |                |     | 0.016                   | 62.5          |
|                |     |                | 0.7 |                |     |                |     | 0.014                   | 71.4          |
|                |     |                | 0.6 |                |     |                |     | 0.012                   | 83.3          |
|                | 0.1 |                | 0.5 | 1:100          | 1.0 |                |     | 0.010                   | 100.0         |
|                |     |                | 0.4 |                | 0.9 |                |     | 0.009                   | 111.1         |
|                |     |                |     |                | 0.8 |                |     | 0.008                   | 125.0         |
|                |     |                | 0.3 |                | 0.7 |                |     | 0.007                   | 142.9         |
|                |     |                |     |                | 0.6 |                |     | 0.006                   | 166.7         |
|                |     |                | 0.2 |                | 0.5 |                |     | 0.005                   | 200.0         |
|                |     |                |     |                | 0.4 |                |     | 0.004                   | 250.0         |
|                |     |                | 0.1 |                | 0.3 |                |     | 0.003                   | 333.3         |
|                |     |                |     |                | 0.2 | 1:500          | 1.0 | 0.002                   | 500.0         |
|                |     |                |     |                |     |                | 0.9 | 0.0018                  | 555.0         |
|                |     |                |     |                |     |                | 0.8 | 0.0016                  | 625.0         |
|                |     |                |     |                |     |                | 0.7 | 0.0014                  | 714.0         |
|                |     |                |     |                |     |                | 0.6 | 0.0012                  | 833.3         |
|                |     |                |     |                |     |                | 0.5 | 0.0010                  | 1,000.0       |
|                |     |                |     |                |     |                | 0.4 | 0.0009                  | 1,250.0       |
|                |     |                |     |                |     |                | 0.3 | 0.0008                  | 1,666.7       |
|                |     |                |     |                |     |                | 0.2 | 0.0007                  | 2,500.0       |
|                |     |                |     |                |     |                | 0.1 | 0.0006                  | 5,000.0       |
|                |     |                |     |                |     |                |     | 0.0005                  | 10,000.0      |
| 1:1,000        | 1.0 |                |     |                |     |                |     |                         |               |
|                |     |                |     |                |     |                |     |                         |               |
|                | 0.1 |                |     |                |     |                |     |                         |               |

evaluating the significance of titer changes, and to determine standard deviations and probable errors.

6. *Technical Precautions to Be Observed.*—

(a) *Cleansing of Pasteur-Chamberland Candles.*—The candles are autoclaved and then cleaned by running tap water through them. They are thoroughly dried and then brought to white heat in a muffle furnace. This last step is essential as the candles are very fine and clog easily. Finally, they are attached to the apparatus and the whole is autoclaved twice before filtering.

(b) *Cleansing and Neutralizing of Glassware.*—All glassware is boiled in ivory soap flakes, neutralized with HCl and rinsed with neutral distilled water.

(c) *Incubation of Streptolysin Broth.*—It has been found necessary to stop the growth of culture at 15 hours to obtain a high titered and stable lysin.

(d) *Centrifuging of Streptolysin Preparatory to Filling.*—For making large amounts of lysin a chilled Sharples centrifuge is used, capable of siphoning off 12 liters of lysin in 30 minutes. The Sharples centrifuge used in this laboratory is easily autoclaved. For small quantities of lysin the International centrifuge holding 250 cc. bottles is used. It is important that both the centrifuge cups and the 250 cc. bottles be chilled before use.

(e) *Reduction of Streptolysin.*—It is desirable to reduce under the same conditions each time as far as possible. Change in temperature influences the strength of lysin; so, although reduction at a higher temperature takes place more rapidly, it is most convenient to pack the flasks in ice and reduce with 29 inch vacuum for 5 hours. It is essential that the normal sodium hydroxide and the powdered sodium hydrosulfite be fresh. Sealing of all connections and openings with a coating of glycerine glue (8) insures complete reduction. Shaking the lysin every 30 minutes during the reduction is also an aid.

(f) *Tubing, Flasking and Sealing of Reduced Lysin.*—Reduced lysin may be most conveniently preserved in 50 cc. Florence flasks or in test tubes. It is quickly and carefully pipetted to avoid bubbles, then sealed to a depth of 2 mm. with warm sterile vaseline or with a sterile mixture of one part paraffin and five parts vaseline. The latter makes a firmer seal. Titrations have demonstrated that there is no difference between lysin which is tubed and sealed the day of reduction or 48 hours later, provided that the flask is well sealed off after reduction. The pH of reduced lysin usually lies between 7.1 and 7.3. If the pH is below 7.0, the titer is lower but the lysin may still be used. It appears that the pH of the more lytic and the more stable filtrates falls between 7.1 and 7.6.

(g) *Pipettes for Titrating.*—Because of the variations in the accuracy of pipettes, calibrated pipettes are employed for the cell suspension, lysin, saline, globulin control and for testing of new lysins. The use of the same pipettes helps to standardize to some extent the tests from day to day. This also applies to the graduates used in making up the lysin.

(h) *Suspension of Cells.*—Defibrinated fresh rabbit's red cells are washed six times with normal salt solution (0.85 per cent) in graduated centrifuge tubes and on the sixth washing packed for 15 minutes at 2,200 revolutions per minute. It is essential in these tests to have the cells free from traces of hemolysis. The

cells are made up to a 5 per cent suspension, using the tube in which they were packed for this purpose.

(i) *Dilutions*.—All dilutions of serum and lysin are made with 0.85 per cent saline, not with broth.

(j) *Titration*.—It is important when titrating sera to do both the preliminary and final titrations on the same day, so as to rule out any possible variability in cell suspension or lysin dilution.

(k) *Rabbits*.—Because of the great variation in the erythrocytes of rabbits, the cells of each new rabbit should be tested for spontaneous hemolysis.

*B. The Natural Level of Antistreptolysin*

In order to determine the natural level of antistreptolysin in human blood, the authors selected for study a group of student nurses entering training at the Presbyterian Hospital in September, 1932. These

TABLE II  
*The Antistreptolysin Titers in Units of a Class of Student Nurses, before and after Exposure to Hospital Infections*

| Those who contracted hemolytic streptococcus infections |              | Those who escaped hemolytic streptococcus infection |             |
|---|--------------|---|-------------|
| Oct., 1932  | Apr., 1934   | Oct., 1932  | Apr., 1934  |
| 33  | 200          | 20  | 20          |
| 33  | 333          | 25  | 25          |
| 50  | 111          | 25  | 33          |
| 83  | 143          | 33  | 33          |
| 83  | 167          | 33  | 33          |
| 83  | 167          | 33  | 33          |
| 83  | 200          | 33  | 33          |
| 100   | 250          | 33  | 50          |
| 100   | 333          | 33  | 63          |
| 167   | 333          | 50  | 33          |
| (Median 83)   | (Median 200) | 50  | 50          |
|   |              | 50  | 63          |
|   |              | 63  | 55          |
|   |              | 83  | 83          |
|   |              | 125   | 83          |
|   |              | 125   | 125         |
|   |              | 167   | 125         |
|   |              | (Median 50)   | (Median 50) |

subjects were kept under clinical observation, and throat cultures were taken during periods of respiratory infection. Antistreptolysin determinations were made at the beginning of the study, at subsequent intervals and again in the spring of 1934 after 18 months of exposure to hospital infections. Thirty members of the class were present throughout the period of observation. The findings in these thirty individuals are classified in three subgroups according to their clinical courses: (a) those who contracted pharyngitis, which was proven on culture to be of hemolytic streptococcus origin; (b) those who contracted other infections, the agent being unknown; (c) those who appeared to escape infection with *Streptococcus hemolyticus*. The findings are shown in Table II.

At the beginning of this study the median value for the thirty individuals was 63 units. Only five subjects had a titer greater than 100 units. Each of the ten subjects infected with hemolytic streptococcus showed a subsequent rise in titer. None of the sixteen subjects who escaped infection with hemolytic streptococcus developed a significant rise in titer. The median titer of this group, who appeared to escape streptococcus infection, was 50 units before entering training and was still 50 units 18 months later. These observations are interpreted to mean that the natural human antistreptolysin value is ordinarily about 50 units.

#### SUMMARY

1. A method for determining the antistreptolysin titer is described in detail.
2. The natural human level of antistreptolysin determined in this way is approximately 50 units.

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STUDIES ON THE IMMUNE RESPONSE OF THE RHEUMATIC SUBJECT AND ITS RELATIONSHIP TO ACTIVITY OF THE RHEUMATIC PROCESS\*

II. OBSERVATIONS ON AN EPIDEMIC OF INFLUENZA FOLLOWED BY HEMOLYTIC STREPTOCOCCUS INFECTIONS IN A RHEUMATIC COLONY

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Several bacteriological studies of outbreaks of acute rheumatism have been made in England. Sheldon (1) observed a series of severe recrudescences among the rheumatic children at Cheyne Hospital in the winter of 1930-31. Collis studied the throat flora of Sheldon's patients and reported (2) that out of thirty-two children with hemolytic streptococcus infections of the pharynx, twenty-four had rheumatic recrudescences. Bradley (3, 4) observed two severe outbreaks of acute rheumatism in English boarding schools, following epidemics of respiratory infections, both in 1929 and in 1931. The strains of hemolytic streptococcus associated with these three outbreaks were studied by Griffith (5). Agglutinin absorption tests showed that within each epidemic, the organisms from a number of patients were serologically identical but that the three epidemic strains were distinct. Collis's outbreak followed infections with the Carter strain (Griffith type 13). Bradley's two outbreaks followed epidemics with the Hutchinson strain in 1929 (Griffith type 18) and the Beatty strain in 1931 (Griffith type 17). Organisms antigenically identical with each of these three strains have been isolated in New York City from patients with pharyngitis, which was followed by severe attacks of acute rheumatism (5, 6). The purpose of the present paper is to

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record observations on a series of infections which occurred during the spring of 1934 at The Pelham Home, an institution for the convalescent care of children with heart disease.

### *The Host Group at The Pelham Home*

The group under observation consisted of thirty-five girls between the ages of 6 and 16 with cardiac disease. One of these had congenital heart disease; the others were known to be rheumatic subjects. The tonsils had been removed in all but four. They had been, with a few exceptions, under the care of the authors for a period of 1 to 6 years. Each had had in the past either a frank rheumatic attack or a number of mild manifestations accompanied by the development of the auscultatory signs of mitral stenosis. Some of them had been at The Pelham Home for a period of several years; others were admitted only a few weeks before the commencement of school in September, 1933, when this particular study began. At that time some of the children had appeared free of rheumatic activity for more than 1 year; others were symptom-free but convalescent from a recent attack; others were experiencing mild manifestations of a subsiding disease process. All appeared in moderately good health. Living conditions were ideal with one exception, the close contact which might facilitate the rapid transmission of infection. The girls attended school in one room of The Pelham Home and slept in three dormitories, containing six, eleven and thirteen beds. They came in contact with their families once each month, and were exposed to the respiratory infections of a teacher and of seven individuals occupying rooms at The Pelham Home—four nurses, a cook, a maid and a janitor. They were all fed on a presumably adequate diet, with ample fresh fruit, fresh vegetables and milk, and sufficient calories to cause a considerable gain in weight in each individual. They were protected from dampness and yet had the opportunity to play almost every afternoon in the fresh air and sunshine. Extreme fluctuations in temperature were obviated by automatic thermostatic control of the building at approximately 70°F. during the day and 55°F. at night. The children were seen and questioned each morning by the nurse in charge of The Pelham Home. Rectal temperatures and pulse rates were recorded each morning and night. The patients received no medication other than cod liver oil. Physical examinations were made weekly, and the throat flora of each patient was cultured twice a week. The progress of the group throughout the year may be divided into two periods: September to March 20, March 20 through July.

### *The Epidemic of Influenza*

Between September, 1933, and January, 1934, the health of the group of thirty children at The Pelham Home was unusually good. Seven individuals were restricted in activity because of evidence pointing to subsiding carditis. Four individuals contracted "colds"

which were not followed by rheumatic manifestations. During early January mild vague rheumatic symptoms were experienced by two individuals, occurring about 10 days after "colds." Eight other children contracted "colds" and four had chicken pox without developing rheumatic recrudescences. There was no evidence of active carditis in any member of the group. Four patients were discharged during the latter part of this period in excellent condition, the disease apparently inactive. The blood sedimentation rates (determined by Westergren's (7) modification of Fåhræus' method) at the end of February were the lowest recorded for any group tested at any time at The Pelham Home (twenty-four below 20, three between 20 and 30 mm.). This corroborated the clinical impression of minimal rheumatic activity in this group of rheumatic children.

The phenomena which led to the second half of this study began on March 15, when the cook became critically ill with a temperature of  $105^{\circ}$  and prostration. The illness resembled epidemic influenza and lasted for 7 days. On March 20, two of the children became ill with this respiratory infection, and during the week eight others were prostrated. The symptoms were almost identical in all instances, consisting of malaise for 1 day followed by a 3 day fever rising to a peak of about  $104^{\circ}\text{F}$ . On the 2nd day the fever was accompanied by nausea, vomiting, aches, cephalalgia and pallor, followed by marked weakness for a few succeeding days. The intensity of the disease gradually diminished. The remainder of the group, with possibly six exceptions, were infected during April; however, the clinical picture in these children was that of a severe "cold" with only slight rise in temperature.

#### *Outbreak of Secondary Infections*

The first three patients to contract influenza recovered from this infection in 3 days. While convalescent, the first two individuals developed pharyngitis and the third tracheitis. The pharyngeal infections were not severe, were characterized by injection of the mucous membranes, edema and a little exudate, which persisted for only 2 or 3 days. Tracheitis was characterized by paroxysms of coughing and hoarseness. Altogether twelve patients, while convalescent from influenza, developed signs or symptoms of secondary

infection of the upper respiratory tract. In one child influenza was followed by severe bronchitis. Except for this patient all of the children were able to return to school 1 week after influenza. Later, two individuals, after having completely recovered from uncomplicated influenza and having been symptom-free for 2 weeks or more, contracted throat infections. Finally, five individuals who had entirely escaped influenza developed severe or mild pharyngitis.<sup>1</sup> During these infections there was only a slight increase in the blood sedimentation rate. An analysis of these respiratory infections revealed first a wave of typical severe influenza beginning precipitously on March 20, gradually becoming milder and complicated by a series of secondary infections of the upper respiratory tract. The earlier cases of secondary infection occurred during recovery from influenza; the later cases appeared independently. Bacteriological studies indicated that these two outbreaks of respiratory infection were due to two distinct agents.

### *Bacteriological Studies*

Studies of the throat flora were conducted on each patient during the entire period of observation. Two cultures were taken each week from the surface of the tonsils or the tonsillar fossae. These were streaked on fresh rabbit blood agar plates; the growth was examined after 24 hours incubation at 37°C. The presence and number of organisms was recorded in uniform manner. All pathogens or potential pathogenic agents were studied in detail. In this way it was possible to know the character of the throat flora harbored by each individual, and it was usually possible to detect the arrival of new organisms at The Pelham Home.

The basic flora in the throats of all the patients at The Pelham Home during the fall months consisted of *Streptococcus viridans*, Gram-negative cocci, Bacillus X, staphylococci, diphtheroid bacilli and anhemolytic streptococci. A few individuals carried Pfeiffer bacillus, pneumococcus or hemolytic streptococcus in small numbers. The throat flora of the group showed no striking change during the winter months. Hemolytic streptococcus appeared in moderate numbers in

<sup>1</sup> Severe pharyngitis occurred in the individuals who had only recently developed rheumatism and in the non-rheumatic subject. The patients with rheumatic heart disease of long standing had extremely mild throat infections.

about one-third of the patients but was not the predominating organism in a single individual. These organisms formed glossy colonies on chocolate agar plates, produced no detectable skin toxin or streptolysin, and all fell into the group of Hemolytic III, according to Holman's classification based on sugar fermentation reactions. They did not appear to be associated with active disease.

At the onset of the epidemic of influenza a filterable virus was recovered from nasal washings (8) although the throat flora showed no changes. However, in the first two patients contracting influenza, hemolytic streptococcus appeared in their throat cultures during convalescence, at the time that they developed pharyngitis. Other individuals had completely recovered from influenza when they developed throat infections, and hemolytic streptococcus appeared in predominance during this pharyngitis. The change in throat flora was easily detected in individuals with infection of the pharynx, but was less definite in the children who developed bronchitis or tracheitis after influenza. In the former hemolytic streptococcus predominated; in the latter the organism was present in small numbers. The organisms associated with these secondary infections appeared to be a single type with the following characteristics: they formed matt (9) colonies on chocolate blood agar plates; were strong streptolysin producers; and fermented lactose and salicin but not mannite, falling into Holman's classification of *Streptococcus pyogenes*. These organisms seemed identical with each other, remained stable in blood broth and were culturally distinct from the other strain (Hemolytic III) which had been carried in the throats of a number of patients during the winter months, which did not produce detectable streptolysin or toxin, and which did not give rise to symptoms. All of the epidemic strains were found in this laboratory to produce strong skin toxins. Two of these, the organisms from Hallahan and Westwater, were studied more closely by Hooker. He tested their filtrates on suitable reactors and observed that both produced strong B toxin and weak A toxin. (See Reference 10.) The Hallahan strain caused a skin reaction 18 x 20 mm. in dilution 1:10,000 and 16 x 18 mm. in 1:15,000, the Westwater strain, 18 x 21 mm. and 18 x 19 mm. in these dilutions. Both toxins in dilution of 1:15,000 were completely neutralized by 1 x 0.1 of 50 units of B containing neutralizing serum.

Hooker<sup>2</sup> considered "the Hallahan and Westwater strains toxigenically indistinguishable."

The following questions relating to the identity of these strains were investigated: (a) whether early and late epidemic strains were of a single serological type; (b) whether the patient with congenital heart disease was infected with the epidemic strain; (c) whether the epidemic strains were of the same serological type as the strain which had been carried in the throats previous to the epidemic; (d) whether the patient who developed pharyngitis and acute rheumatism shortly after discharge from The Pelham Home had been infected with the epidemic strain. Lancefield's (11) technique was employed for this study. This author has demonstrated that:

"HCl extracts of *Streptococcus hemolyticus* contain type-specific, as well as non-type-specific substances. The type-specific substance may be detected by the use of antibacterial sera absorbed with heterologous strains of hemolytic streptococcus. Such absorbed sera are type-specific. They are precipitated only by extracts of strains of the homologous types."

By the use of the precipitin test with absorbed serum it is possible to divide hemolytic streptococci into distinct serological types. The technique used in the present study was as follows:

(a) *Preparation of HCl Extracts.*—The extracts were prepared according to Lancefield's technique.

(b) *Technique of Immunization.*—The rabbit antisera were prepared as follows: Adult animals weighing between 2 and 3 kilos were first tested to rule out the presence of natural antibodies to streptococcus. They were immunized with heat-killed and then with living organisms over a period of 4 weeks. The organisms were obtained from 0.1 per cent dextrose broth, were washed in saline and preserved in merthiolate 1:10,000. A test bleeding was made on the 4th day after the last injection, and the animals killed on the 5th day. Four rabbits were immunized with each organism. The schedule is given on page 143.

The last six doses were supplemented with living 15 hour broth cultures. The broth was removed, culture washed and brought up to volume with saline and given in doses of 0.05 cc. at first and 0.1 cc. in the last four injections.

(c) *Absorption of Sera.*—Absorption of sera was carried out as follows: Bacteria from 1.5 liters of plain broth culture of heterologous (S 24, supplied by Dr. Lancefield) and a similar quantity of homologous organisms were centrifuged. The organisms in each instance were washed twice with saline, killed by heating at 58°C. and suspended in 2 cc. of saline for 1 hour. To each tube of bacteria 4 cc. of serum were added drop by drop and the mixture thoroughly shaken. The mixture was incubated in a 37° bath for 30 minutes, refrigerated for 3 hours, centrifuged and the serum removed. This serum was again added in the same way to a fresh suspension of bacteria. Absorption was repeated as before. The supernatant serum was then tested. Absorption was found complete in each instance.

<sup>2</sup> Personal communication.

| Wk. 1, Day 1 | Subcutaneous injection | 0.5 cc. killed culture, 5 billion per cc. |
|--------------|------------------------|---|
| " 1, " 2     | " "                    | 0.5 " " " 5 " " "                         |
| " 1, " 3     | " "                    | 0.5 " " " 5 " " "                         |
| " 1, " 4     | Intraperitoneal "      | 1.0 " " " 1 " " "                         |
| " 1, " 5     | " "                    | 1.0 " " " 1 " " "                         |
| " 1, " 6     | " "                    | 1.0 " " " 1 " " "                         |
| " 2, " 1     | Intravenous "          | 0.5 " " " 1 " " "                         |
| " 2, " 2     | " "                    | 0.5 " " " 1 " " "                         |
| " 2, " 3     | " "                    | 1.0 " " " 1 " " "                         |
| " 2, " 4     | " "                    | 1.0 " " " 1 " " "                         |
| " 3, " 1     | " "                    | 2.0 " " " 2 " " "                         |
| " 3, " 2     | " "                    | 2.0 " " " 2 " " "                         |
| " 3, " 3     | " "                    | 1.0 " " " 1 " " "                         |
| " 3, " 4     | " "                    | 1.0 " " " 1 " " "                         |
| " 4, " 1     | " "                    | 0.9 " " " 1 " " "                         |
| " 4, " 2     | " "                    | 0.9 " " " 1 " " "                         |
| " 4, " 3     | " "                    | 0.9 " " " 1 " " "                         |
| " 4, " 4     | " "                    | 0.9 " " " 1 " " "                         |

Eight strains of hemolytic streptococcus were selected for this study. All of them were isolated during the first 48 hours of acute pharyngitis when they were present in large numbers in the throat cultures. Two strains, EpEH and EpEW obtained from Hallahan (page 166) and Westwater (page 164) were from the first two patients to become infected shortly after the outbreak of influenza. Three strains, EpEF, EpER and EpEP were obtained from Fay (page 165), Raimonde (page 164) and Patterson (page 168) who contracted pharyngitis entirely independent of influenza. Strain NEP, glossy, was present in the throat of Patterson previous to the influenza epidemic and was representative of the Hemolytic III organisms carried by a number of patients during the winter months. Strain EpS was cultured from the throat of Sucich (page 173) during pharyngitis contracted at the time that strains EpER and EpEP were recovered. Strain E 112, matt, was cultured from the throat of Ferrara (page 149) during pharyngitis which developed 2 weeks after the patient was discharged from The Pelham Home. The results of the precipitin tests of strains from the first two and last two patients to be infected during the epidemic and of two strains not associated with the epidemic are presented in Table I A, B and C. With the exception of EpER all of the rabbit sera were of unusually good titer.

From the data presented in Table I A, B and C it is seen that precipitin reactions occurred between HCl extracts of the Ep (epidemic) strains and their unabsorbed and heterologously absorbed sera. There were no reactions between their HCl extracts and their homologously

Hooker<sup>2</sup> considered "the Hallahan and Westwater strains toxigenically indistinguishable."

The following questions relating to the identity of these strains were investigated: (a) whether early and late epidemic strains were of a single serological type; (b) whether the patient with congenital heart disease was infected with the epidemic strain; (c) whether the epidemic strains were of the same serological type as the strain which had been carried in the throats previous to the epidemic; (d) whether the patient who developed pharyngitis and acute rheumatism shortly after discharge from The Pelham Home had been infected with the epidemic strain. Lancefield's (11) technique was employed for this study. This author has demonstrated that:

"HCl extracts of *Streptococcus hemolyticus* contain type-specific, as well as non-type-specific substances. The type-specific substance may be detected by the use of antibacterial sera absorbed with heterologous strains of hemolytic streptococcus. Such absorbed sera are type-specific. They are precipitated only by extracts of strains of the homologous types."

By the use of the precipitin test with absorbed serum it is possible to divide hemolytic streptococci into distinct serological types. The technique used in the present study was as follows:

(a) *Preparation of HCl Extracts.*—The extracts were prepared according to Lancefield's technique.

(b) *Technique of Immunization.*—The rabbit antisera were prepared as follows: Adult animals weighing between 2 and 3 kilos were first tested to rule out the presence of natural antibodies to streptococcus. They were immunized with heat-killed and then with living organisms over a period of 4 weeks. The organisms were obtained from 0.1 per cent dextrose broth, were washed in saline and preserved in merthiolate 1:10,000. A test bleeding was made on the 4th day after the last injection, and the animals killed on the 5th day. Four rabbits were immunized with each organism. The schedule is given on page 143.

The last six doses were supplemented with living 15 hour broth cultures. The broth was removed, culture washed and brought up to volume with saline and given in doses of 0.05 cc. at first and 0.1 cc. in the last four injections.

(c) *Absorption of Sera.*—Absorption of sera was carried out as follows: Bacteria from 1.5 liters of plain broth culture of heterologous (S 24, supplied by Dr. Lancefield) and a similar quantity of homologous organisms were centrifuged. The organisms in each instance were washed twice with saline, killed by heating at 58°C. and suspended in 2 cc. of saline for 1 hour. To each tube of bacteria 4 cc. of serum were added drop by drop and the mixture thoroughly shaken. The mixture was incubated in a 37° bath for 30 minutes, refrigerated for 3 hours, centrifuged and the serum removed. This serum was again added in the same way to a fresh suspension of bacteria. Absorption was repeated as before. The supernatant serum was then tested. Absorption was found complete in each instance.

<sup>2</sup> Personal communication.

TABLE I—*Concluded*  
C

| HCl extract        | Rabbit serum NEP |                      |              | Rabbit sera       | HCl extract NEP |                      |              |
|--------------------|------------------|----------------------|--------------|-------------------|-----------------|----------------------|--------------|
|                    | Unabsorbed       | Absorbed with strain |              |                   | Unabsorbed      | Absorbed with strain |              |
|                    |                  | Homologous           | Heterologous |                   |                 | Homologous           | Heterologous |
| NEP                | + + + + +        | - - -                | + + + + +    | NEP               | + + + + +       | - - -                | + + + + +    |
| EpEH               | -                | - - -                | -            | EpEH              | -               | - - -                | -            |
| EpEW               | -                | - - -                | -            | EpEW              | -               | - - -                | -            |
| EpER               | -                | - - -                | -            | EpER              | -               | - - -                | -            |
| EpEF               | -                | - - -                | -            | EpEF              | -               | - - -                | -            |
| EpEP               | -                | - - -                | -            | EpEP              | -               | - - -                | -            |
| EpS                | -                | - - -                | -            | EpS               | -               | - - -                | -            |
| E 112              | -                | - - -                | -            | E 112             | -               | - - -                | -            |
| Rabbit serum E 112 |                  |                      |              | HCl extract E 112 |                 |                      |              |
| E 112              | - + + +          | - - -                | - + + +      | E 112             | - + + +         | - - -                | - + + +      |
| EpEH               | -                | - - -                | -            | EpEH              | -               | - - -                | -            |
| EpEW               | -                | - - -                | -            | EpEW              | -               | - - -                | -            |
| EpER               | -                | - - -                | -            | EpER              | -               | - - -                | -            |
| EpEF               | -                | - - -                | -            | EpEF              | -               | - - -                | -            |
| EpEP               | -                | - - -                | -            | EpEP              | -               | - - -                | -            |
| EpS                | -                | - - -                | -            | EpS               | -               | - - -                | -            |
| NEP                | -                | - - -                | -            | NEP               | -               | - - -                | -            |

absorbed sera. The absence of specific reactions between the sera and HCl extracts of Ep strains and the extracts or sera of the other organisms clearly defined hemolytic streptococcus EpEH, EpEW, EpER, EpEF, EpEP and EpS as belonging to one group. Likewise the absence of reactions showed that Strain NEP and Strain E 112 were each antigenically different from the epidemic strains.

The results of the tests are believed to indicate: (a) that all of the epidemic strains, including both those isolated from infections during influenza (Table I A) and those isolated from infections independent of influenza (Table I B) were a single strain; (b) that Strain EpS, infecting a non-rheumatic subject, was indistinguishable from the other five epidemic strains; (c) that all of the epidemic strains were antigenically distinct from the Strain NEP (Table I C), which had been prevalent before the onset of the epidemic; (d) that Strain E 112 (Table I C) was antigenically different, both from the six epidemic strains and also



TABLE I

*Precipitin Reactions of Strains of Hemolytic Streptococcus Recovered from Pelham Home Patients*

A

| HCl extract       | Rabbit serum EpEH (Diluted 1:1) |                      |                   | Rabbit sera      | HCl extract EpEH |                      |                   |
|-------------------|---------------------------------|----------------------|-------------------|------------------|------------------|----------------------|-------------------|
|                   | Unabsorbed                      | Absorbed with strain |                   |                  | Unabsorbed       | Absorbed with strain |                   |
|                   |                                 | Homolo-<br>gous      | Heterolo-<br>gous |                  |                  | Homolo-<br>gous      | Heterolo-<br>gous |
| EpEH              | + + + + +                       | - - -                | + + + + +         | EpEH             | + + + + +        | - - -                | + + + + +         |
| EpEW              | + + + + +                       | - - -                | - + +             | EpEW             | - ± +            | - - -                | - ± +             |
| EpER              | + + +                           | - - -                | - - -             | EpER             | - - -            | - - -                | - - -             |
| EpEF              | - - + +                         | - - -                | - - + +           | EpEF             | - ± + +          | - - -                | - ± + +           |
| EpEP              | - + + + +                       | - - -                | - + + + +         | EpEP             | - + + + +        | - - -                | - + + + +         |
| EpS               | - + +                           | - - -                | - ± ±             | EpS              | + - +            | - - -                | - - +             |
| NEP               | - - -                           | - - -                | - - -             | NEP              | - - -            | - - -                | - - -             |
| E 112             | - - -                           | - - -                | - - -             | E 112            | - - -            | - - -                | - - -             |
| Rabbit serum EpEW |                                 |                      |                   | HCl extract EpEW |                  |                      |                   |
| EpEW              | + + +                           | - - -                | - + +             | EpEW             | + + +            | - - -                | - + + +           |
| EpEH              | - ± +                           | - - -                | - ± +             | EpEH             | + + + +          | - - -                | - + +             |
| EpER              | - - ±                           | - - -                | - - ±             | EpER             | - - -            | - - -                | - - -             |
| EpEF              | ± + +                           | - - -                | - ± +             | EpEF             | + + +            | - - -                | - + +             |
| EpEP              | - ± +                           | - - -                | - - +             | EpEP             | + + + +          | - - -                | - + + +           |
| EpS               | - - ±                           | - - -                | - - ±             | EpS              | + - +            | - - -                | - - -             |
| NEP               | - - -                           | - - -                | - - -             | NEP              | - - -            | - - -                | - - -             |
| E 112             | - - -                           | - - -                | - - -             | E 112            | - - -            | - - -                | - - -             |

B

|                  | Rabbit serum EpEP |       |           |                 | HCl extract EpEP |       |           |
|------------------|-------------------|-------|-----------|-----------------|------------------|-------|-----------|
| EpEP             | + + + + +         | - - - | - - + +   | EpEP            | + + + + +        | - - - | - - + +   |
| EpEH             | - + + + +         | - - - | - + + +   | EpEH            | - + + + +        | - - - | - + + + + |
| EpEW             | + + + +           | - - - | - + + +   | EpEW            | - ± +            | - - - | - ± +     |
| EpER             | - ± +             | - - - | - - -     | EpER            | - - -            | - - - | - - -     |
| EpEF             | ± + + +           | - - - | - + + +   | EpEF            | - ± +            | - - - | - ± +     |
| EpS              | - + +             | - - - | - - ±     | EpS             | - - -            | - - - | - - -     |
| NEP              | - - +             | - - - | - - -     | NEP             | - - -            | - - - | - - -     |
| E 112            | - - -             | - - - | - - -     | E 112           | - - -            | - - - | - - -     |
| Rabbit serum EpS |                   |       |           | HCl extract EpS |                  |       |           |
| EpS              | + + + + +         | - - - | - + + + + | EpS             | + + + + +        | - - - | - + + + + |
| EpEH             | + - +             | - - - | - - ±     | EpEH            | - + +            | - - - | - ± ±     |
| EpEW             | + - +             | - - - | - - -     | EpEW            | - - -            | - - - | - - -     |
| EpER             | + + + +           | - - - | - + + +   | EpER            | - + + + +        | - - - | - + + +   |
| EpEF             | + - +             | - - - | - ± + +   | EpEF            | - - ±            | - - - | - - ±     |
| EpEP             | - - -             | - - - | - - -     | EpEP            | - + +            | - - - | - - ±     |
| NEP              | - - -             | - - - | - - -     | NEP             | - - -            | - - - | - - -     |
| E 112            | - - -             | - - - | - - -     | E 112           | - - -            | - - - | - - -     |

The determinations indicate three readings; first, made at 20 minutes at room temperature; second, after 2 hours incubation at 37.5°C.; third, after 18 hours in the ice box, not centrifuged.

TABLE I—*Concluded*  
C

| HCl extract        | Rabbit serum NEP |                      |              | Rabbit sera       | HCl extract NEP |                      |              |
|--------------------|------------------|----------------------|--------------|-------------------|-----------------|----------------------|--------------|
|                    | Unabsorbed       | Absorbed with strain |              |                   | Unabsorbed      | Absorbed with strain |              |
|                    |                  | Homologous           | Heterologous |                   |                 | Homologous           | Heterologous |
| NEP                | +++              | ---                  | +++          | NEP               | +++             | ---                  | +++          |
| EpEH               | -                | -                    | -            | EpEH              | -               | -                    | -            |
| EpEW               | -                | -                    | -            | EpEW              | -               | -                    | -            |
| EpER               | -                | -                    | -            | EpER              | -               | -                    | -            |
| EpEF               | -                | -                    | -            | EpEF              | -               | -                    | -            |
| EpEP               | -                | -                    | -            | EpEP              | -               | -                    | -            |
| EpS                | -                | -                    | -            | EpS               | -               | -                    | -            |
| E 112              | -                | -                    | -            | E 112             | -               | -                    | -            |
| Rabbit serum E 112 |                  |                      |              | HCl extract E 112 |                 |                      |              |
| E 112              | - ++             | ---                  | - ++         | E 112             | - ++            | ---                  | - ++         |
| EpEH               | -                | -                    | -            | EpEH              | -               | -                    | -            |
| EpEW               | -                | -                    | -            | EpEW              | -               | -                    | -            |
| EpER               | -                | -                    | -            | EpER              | -               | -                    | -            |
| EpEF               | -                | -                    | -            | EpEF              | -               | -                    | -            |
| EpEP               | -                | -                    | -            | EpEP              | -               | -                    | -            |
| EpS                | -                | -                    | -            | EpS               | -               | -                    | -            |
| NEP                | -                | -                    | -            | NEP               | -               | -                    | -            |

absorbed sera. The absence of specific reactions between the sera and HCl extracts of Ep strains and the extracts or sera of the other organisms clearly defined hemolytic streptococcus EpEH, EpEW, EpER, EpEF, EpEP and EpS as belonging to one group. Likewise the absence of reactions showed that Strain NEP and Strain E 112 were each antigenically different from the epidemic strains.

The results of the tests are believed to indicate: (a) that all of the epidemic strains, including both those isolated from infections during influenza (Table I A) and those isolated from infections independent of influenza (Table I B) were a single strain; (b) that Strain EpS, infecting a non-rheumatic subject, was indistinguishable from the other five epidemic strains; (c) that all of the epidemic strains were antigenically distinct from the Strain NEP (Table I C), which had been prevalent before the onset of the epidemic; (d) that Strain E 112 (Table I C) was antigenically different, both from the six epidemic strains and also

from the carrier strain. Classification of these six epidemic strains by the use of Lancefield's (11) technique showed that they all fell into our (6) group VI, Griffith's Type 2b, and were antigenically different from the English epidemic strains (page 137). E 112 was indistinguishable from Griffith's Beatty strain, Type 17, our group V.

In summary, the bacteriological study of the throat flora at The Pelham Home showed the presence of two pathogenic agents operating among an isolated group of rheumatic children. These individuals had experienced good health for a long period of time before the onset of infection. A few of them were carriers of an apparently non-pathogenic strain of hemolytic streptococcus before the epidemic, and in two of these there had occurred mild recrudescences following "colds;" however, these children had recovered by the middle of March. At that time there occurred an outbreak of typical influenza, which waned in its intensity but which nevertheless during a period of 3 weeks incapacitated all but four of the thirty children. This infection was probably due to the filterable virus recovered from Raimonde (8). Within a week after the appearance of the influenza a new strain of hemolytic streptococcus appeared at The Pelham Home. It was easily recognized on the 1st day of its manifestations. Unlike the carrier strain, it was associated with acute respiratory infection. At first it seemed to be transmitted only with the help of influenza. At the end of 2 weeks it appeared in the throats of contacts who had escaped influenza, where it caused severe infections. At this time the strain seemed highly communicable and independent of the filterable virus. Four weeks after the subsidence of influenza, hemolytic streptococcus was present in the throat flora of all but two of the group. The serological tests showed that the epidemic (Ep) strains were of a single type, and were antigenically different both from the non-effective (NE) carrier strain and from another effective (E)<sup>3</sup> strain acquired by a Pelham Home patient shortly after arrival in New York City.

<sup>3</sup> The term "effective" has been applied by the authors to strains of hemolytic streptococcus recovered from throat infections which were followed by rheumatic recrudescences in susceptible subjects. Detailed studies on the characteristics of effective (E) and non-effective (NE) strains are to be reported in Paper IV of this series (*J. Clin. Inv.*, 1935, in press).

*The Epidemic of Rheumatic Fever*

Suddenly on April 10, 3 weeks after the onset of influenza, three patients in adjoining beds showed a marked rise in body temperature. All of these individuals had recovered from influenza and secondary infections by April 1. The cause of pyrexia was definitely established during the 2nd week of April, when each of these three children developed fulminating pancarditis. A few days later seven others of the group who had had streptococcus infections changed from a state of

TABLE II

*The Relationship of Respiratory Infections to the Development of Rheumatic Recrudescences*

| Type of respiratory infection   | No. of cases | Type of rheumatic attack | No. of cases |
|---|--------------|--------------------------|--------------|
| No respiratory infection  | 3            | None                     | 3            |
| Influenza without hemolytic streptococcus infection                       | 4            | None                     | 4            |
| Influenza complicated by hemolytic streptococcus infection                | 11           | Severe attacks           | 7            |
|   |              | Mild attacks             | 2*           |
|   |              | No attack                | 2            |
| Influenza followed after an interval by hemolytic streptococcus infection | 2            | Severe attacks           | 2            |
| Hemolytic streptococcus infection without influenza                       | 5            | Severe attacks           | 2            |
|   |              | Mild attacks             | 2            |
|   |              | No attack                | 1†           |

\* One atypical attack.

† Non-rheumatic subject.

good health to one of critical illness. Each of these rheumatic attacks began between 10 and 14 days after the subsidence of the secondary infection. During the latter half of April and early part of May five more children, all but one of whom had had influenza, contracted throat infections. Four of them developed rheumatic attacks between May 8 and May 18. The fifth, a non-rheumatic subject with congenital heart disease, remained symptom-free. One more case of pharyngitis developed on May 15, and was followed by a severe rheumatic attack in June. Three of the 6 attacks beginning in May and

June appeared from 22 to 24 days after the throat infection; however, in one case the pulse, in another the sedimentation rate, in the third the antistreptolysin titer, had been elevated for 10 to 14 days in advance of acute symptoms. Three children who had escaped all respiratory infections and four others who had contracted only influenza, did not develop rheumatic fever. The relationship of these infections to rheumatic recrudescences is summarized in Table II.

TABLE III

*Antistreptolysin Titers (in Units) of Twenty Children at The Pelham Home*

| Patient         | Fall, 1933 | Winter, 1934 |
|-----------------|------------|--------------|
| Allen, G.....   | 143        | 125          |
| Carroll.....    | 125        | 125          |
| Cleary.....     | 143        | 125          |
| Curnan.....     | 71         | 50           |
| Fay.....        | 33         | 33           |
| Ferrara.....    | 63         | 63           |
| Goch.....       | 71         | 63           |
| Gross.....      | 56         | 33           |
| Gunning*.....   | 83         | 200          |
| Hudson.....     | 200        | 200          |
| Kienzle*.....   | 167        | 250          |
| Kiernan.....    | 143        | 125          |
| Libera.....     | 143        | 143          |
| Marten.....     | 56         | 56           |
| Patterson.....  | 143        | 71           |
| Raimonde.....   | 500        | 143          |
| Ryan.....       | 167        | 125          |
| Torres.....     | 167        | 143          |
| Westwater.....  | 125        | 100          |
| Williamson..... | 71         | 63           |

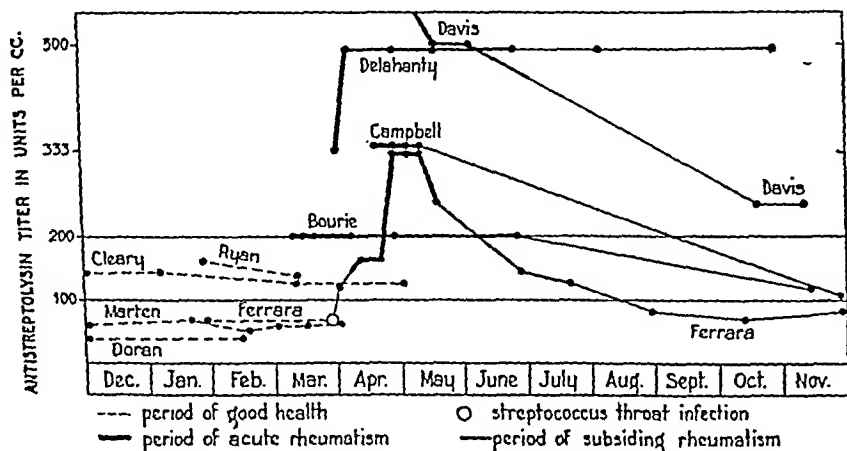
\* These were the two children who had mild recrudescences in February, 1934 about 6 weeks before the epidemic.

### *Immunological Studies*

The findings to be presented in a later paper of this series show a close relationship between hemolytic streptococcus infection and a rise<sup>4</sup> of the antistreptolysin titer in the blood serum. In this con-

<sup>4</sup> The development of antibody to hemolytic streptococcus coincident with the onset of the rheumatic attack has been pointed out in England (12) (precipitins) and by the authors (13) (antistreptolysin).

nection the authors were particularly interested in following the titer of this antibody in the group at The Pelham Home. During the 6 months period of observation previous to the epidemic, there had been a downward trend in the antistreptolysin titer of all but two individuals. These determinations are shown in Table III. The patients discharged in good health just before or during the epidemic had low titers (Ryan, Cleary, Marten and Doran). Those who were admitted in their places had active rheumatism and high titers (Davis, Delahanty, Campbell and Bourie). One individual (Ferrara) was infected with hemolytic streptococcus shortly after discharge from The Pelham Home and readmitted with acute rheumatism.<sup>5</sup> The antistreptolysin curves are presented in Text-fig. 1. The antistreptolysin titer levels changed in many of The Pelham Home children following the outbreak of hemolytic streptococcus infections. For purposes of comparison the patients' titers will be considered in six groups: (1) those who escaped influenza and streptococcus infection; (2) those who contracted influenza and escaped streptococcus infection; (3) those who contracted streptococcus infection with or without influenza but who escaped typical rheumatic attacks; (4) those who contracted strep-



TEXT-FIG. 1. Patients discharged from The Pelham Home before the epidemic and new patients admitted to The Pelham Home during the epidemic.

<sup>5</sup> This observation has been made each year in the group of children returning to their families in New York City during the late winter months.

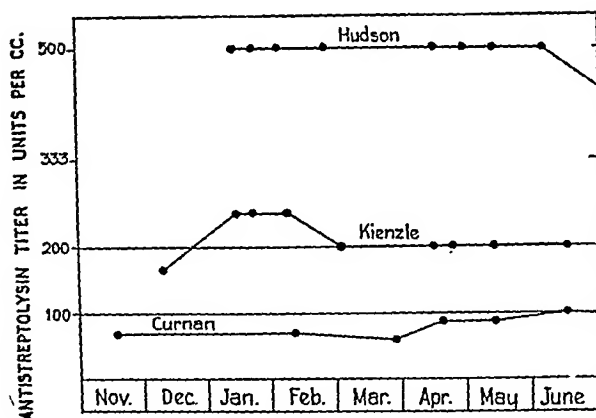
tococcus infection with or without influenza and developed mild rheumatic attacks; (5) those who contracted streptococcus infection while convalescent from influenza and developed severe rheumatic attacks; (6) those who contracted streptococcus infection independent of influenza and developed severe rheumatic attacks.

1. *Antistreptolysin Titers of Patients Escaping Influenza and Streptococcus Infection.*—Three individuals escaped respiratory infection during the spring months at The Pelham Home. One patient, Hudson, had been admitted with subsiding rheumatism in January. The other two patients, Kienzle and Curnan, had been at The Pelham Home for a period of years. The titer levels showed little or no change following the epidemic, and the patients remained in excellent health. The titer curves are presented in Text-fig. 2.

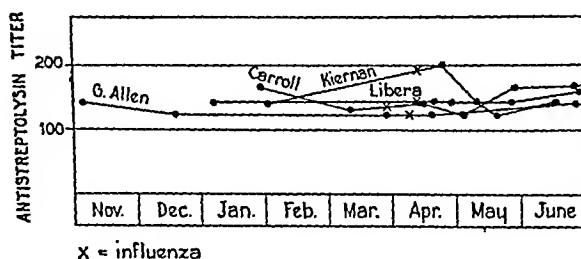
2. *Antistreptolysin Titers of Patients Contracting Influenza and Escaping Streptococcus Infection.*—Four individuals contracted influenza but escaped streptococcus infection. All had been at The Pelham Home for a long period of time. The titer levels showed little or no change following the epidemic. This was in accord with previous observations on rheumatic patients recovering from influenza. After recovery from influenza the patients remained in excellent health. The titer curves are presented in Text-fig. 3.

3. *Antistreptolysin Titers of Patients Contracting Streptococcus Infection but Escaping Acute Rheumatism.*—Four individuals contracted streptococcus infection but failed to develop typical rheumatic attacks. In three of them the streptococcus infections followed influenza. One patient, Sucich, was a non-rheumatic subject; another, McMahan, was an atypical rheumatic subject. There were only moderate rises in antistreptolysin titer. The responses of these individuals will be considered in the following paper. The antistreptolysin curves are presented in Text-fig. 4.

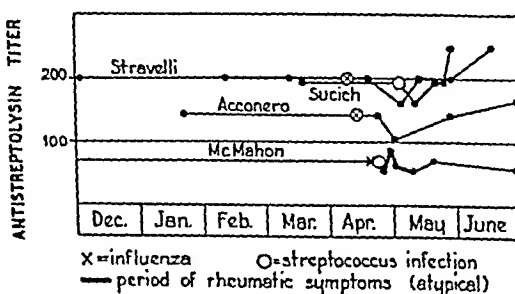
4. *Antistreptolysin Titers of Patients Contracting Streptococcus Infection with or without Influenza and Developing Mild Rheumatic Attacks.*—Three individuals contracted streptococcus infections which were followed by mild rheumatism. Patients Grabeck and Gross had escaped influenza. All three children developed a definite rise in antistreptolysin titer with the onset of rheumatic symptoms. The titer curves are presented in Text-fig. 5.



TEXT-FIG. 2. Three patients who contracted no infection at all during the epidemic.

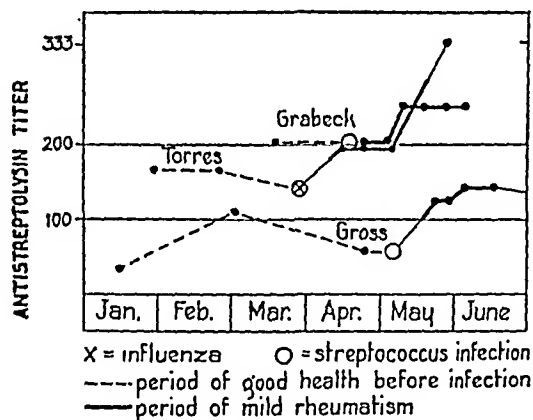


TEXT-FIG. 3. Four patients who contracted influenza which was not followed by hemolytic streptococcus infection.



TEXT-FIG. 4. Four patients who did not develop typical rheumatic fever following infection with hemolytic streptococcus.



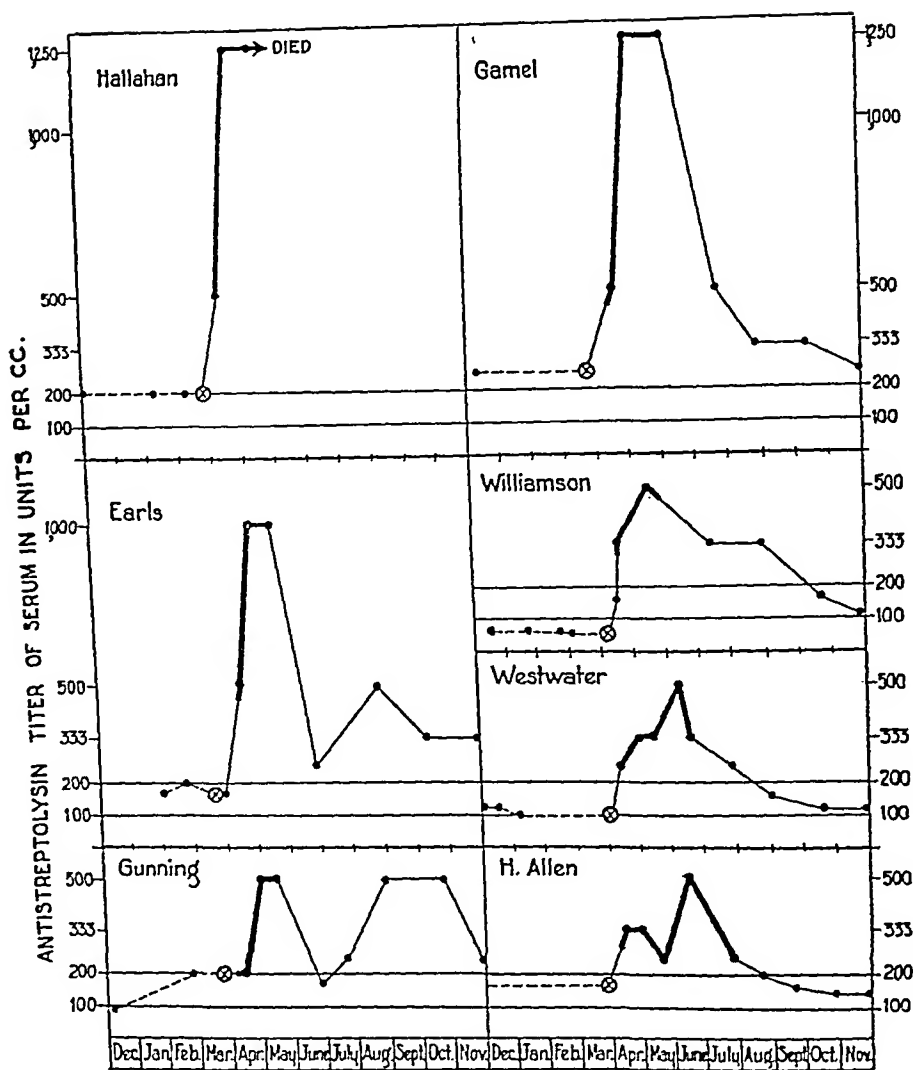


TEXT-FIG. 5. Three patients with mild attacks.

5. *Antistreptolysin Titers of Patients Who Contracted Both Influenza and Streptococcus Infection and Then Developed Severe Rheumatic Attacks.*—Seven patients contracted influenza which was complicated by streptococcus infection. All developed severe rheumatic attacks. In every case, the antistreptolysin titer showed a marked rise at the onset of rheumatic manifestations. The titer curves are presented in Text-fig. 6.

These seven patients developed rheumatic attacks in rapid succession. Each was preceded by upper respiratory tract infections. The infections appeared to be due to two distinct agents—first, a filterable virus causing influenza; second, hemolytic streptococcus causing a variety of respiratory symptoms. In those individuals who developed infection of the pharynx while recovering from influenza, it was possible to see the inflammatory reaction and to obtain hemolytic streptococcus in almost pure culture. In others with tracheitis and bronchitis, the organisms were also recovered but in small numbers. Each patient developed a marked rise in antistreptolysin titer. However, in none of these instances could influenza be eliminated as a possible causative agent in the subsequent severe attacks of pancarditis.

6. *Antistreptolysin Titers of Patients Contracting Streptococcus Infections Independent of Influenza and Developing Severe Rheumatic Attacks.*—Four patients who had been at The Pelham Home throughout the period of this study contracted streptococcus infection in



x = influenza

O = streptococcus infection

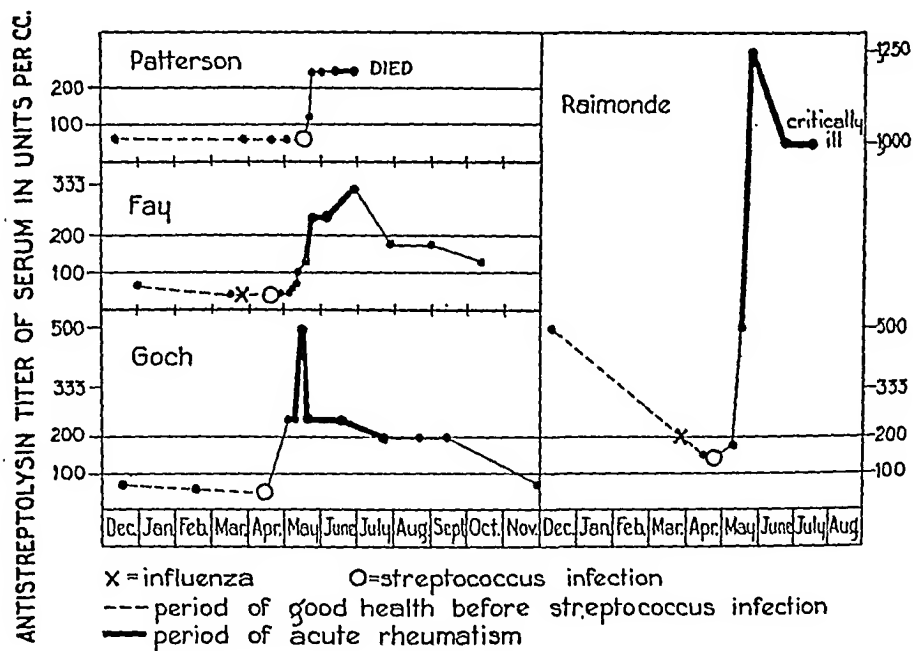
----- period of good health before streptococcus infection

———— period of acute rheumatism

TEXT-FIG. 6. Seven patients with severe attacks following hemolytic streptococcus infection coincident with influenza.

April or May. In each instance this infection occurred independently of influenza. All developed rheumatic attacks of great severity. The onset of these attacks was either accompanied by or just preceded by a sharp rise in antistreptolysin titer. The titer curves are presented in Text-fig. 7.

These four patients contracted hemolytic streptococcus pharyngitis after the influenza epidemic had subsided. Two of these children, Raimonde and Fay, had recovered from influenza during the last week



TEXT-FIG. 7. Four patients with severe attacks following hemolytic streptococcus infection independent of influenza.

in March. The others, Goch and Patterson,<sup>6</sup> had escaped influenza but contracted hemolytic streptococcus pharyngitis at a later date. These infections were all followed by severe rheumatic attacks. One was fatal; and it is doubtful whether the other three individuals will recover. The antistreptolysin titer remained level following influenza and did not rise until the 2nd week after streptococcus infection. From patient Raimonde a filterable virus was recovered (8) during

<sup>6</sup> Patterson's antistreptolysin titer rose to 250 units at the onset of the rheumatic attack. A blood sample was not obtained during the 2nd week of the illness because of her critical condition.

influenza, and hemolytic streptococcus was not present in the throat cultures. Four weeks later the antistreptolysin titer had remained stationary. Then during pharyngitis, hemolytic streptococcus was predominant in the throat flora. This infection was followed after 2 weeks by a rise in antistreptolysin titer and acute rheumatism. Likewise the antistreptolysin determinations in the other patients corroborated the clinical and bacteriological findings, which indicated that influenza was not the etiological agent involved in this outbreak of rheumatic attacks.

#### DISCUSSION

This epidemic infection made it possible to observe the mass response of an isolated colony of rheumatic subjects to distinct pathogenic agents. That the chicken pox virus alone did not activate the rheumatic process was definite. Four children, all highly susceptible subjects, contracted chicken pox during the winter months. The throat flora remained free of hemolytic streptococcus; the antistreptolysin levels remained constant; no patient developed a single rheumatic manifestation. The influence of influenza was at first difficult to assess. The early cases of acute rheumatism had all had influenza accompanied by activity of the secondary invader, hemolytic streptococcus. All developed severe rheumatic attacks coincident with a sharp rise in antistreptolysin titer. From these patients it was not possible to exclude the influenza virus as a possible causative agent. However, other patients who contracted influenza at the same time and who escaped streptococcus infection responded differently. The antistreptolysin titer did not rise, and the rheumatic process remained quiescent in these individuals. Furthermore, two of these individuals subsequently became infected with the epidemic strain of hemolytic streptococcus, as late as 1 month after recovery from influenza. These streptococcus infections were then followed by a sharp rise in antistreptolysin titer and severe rheumatic attacks. Finally, two patients who had escaped influenza, yet who contracted pharyngitis with hemolytic streptococcus, developed fulminating rheumatic attacks with rapid death in one instance. For these reasons it seemed that influenza was significant, so far as rheumatism was concerned, only in facilitating the spread of streptococcus infection.

The character of the streptococcus infections changed during the

course of the epidemic. At first they were all mild and difficult to distinguish clinically from influenza. Some of the later infections were well defined and severe. These occurred in individuals who had previously experienced not more than one rheumatic attack. Whether the severity of the local infection was increased as a result of passage of the strain of streptococcus was unknown; nevertheless it was seen that the organism's effectiveness in initiating rheumatic activity was not altered. Streptococcus infections closely associated with influenza were neither more nor less effective in initiating rheumatic activity than uncomplicated pharyngitis. As influenza subsided, the epidemic strain of streptococcus was transmitted independently.

Finally, the cultural and serological tests showed that the outbreak of acute pharyngitis was due to a single strain of hemolytic streptococcus. Although it was not possible to trace the origin of this strain,<sup>7</sup>

<sup>7</sup> Since writing these papers the authors have had an unusually good opportunity to trace the origin and spread of one streptococcus infection, and to analyze the development of an immune response and the initiation of rheumatic activity. This observation may be summarized briefly.

Patient D. was admitted to The Pelham Home October, 1934, with subsiding rheumatic fever, large tonsils and hemolytic streptococcus in the throat flora. She carried this organism during the winter months apparently without spreading infection. On Apr. 20, 1935, she contracted the common cold, then prevalent at Pelham Home, and was kept in bed at one end of the ward because of severe coughing. On Apr. 22 hemolytic streptococcus increased in her throat flora to almost pure culture. The antistreptolysin titer was 333 units at this time and remained constant for 2 months. The patient escaped a rheumatic recrudescence.

Patient E. was also at Pelham Home during the same period, had no tonsils and no hemolytic streptococci in the throat flora. She contracted the common cold but escaped a rheumatic attack in April, 1935. However, her bed was next to the one occupied by D. On Apr. 26, E. contracted acute pharyngitis with hemolytic streptococcus in almost pure culture. The organism was indistinguishable from that of D (both Griffith Type I). E.'s antistreptolysin titer which had been 250 units, rose to 333 units on May 1, coincident with the development of a recrudescence. 2 weeks later there was another rise to 500 units, and the patient was transferred to Babies Hospital with rheumatic carditis of moderate severity.

It is the authors' opinion that the common cold was probably responsible for the change described in D.'s throat flora, for the severe coughing, and thus for the spreading of D.'s strain to E. The failure of D. to develop a rheumatic attack in the absence of antistreptolysin rise, and the development of a severe recrudescence coincident with the rise in circulating antibody in E. are in accord with the

it was antigenically distinct from the non-effective strain which had been carried in the throat flora previous to the epidemic.

#### SUMMARY

The observations presented in this paper may be summarized as follows:

A study has been made on an isolated group of children with heart disease.

All of these individuals, with one exception, were rheumatic subjects.

Many of them carried a strain of hemolytic streptococcus in the throat flora during the winter of 1934. The organism produced no detectible toxin and was not associated with respiratory disease.

Four patients contracted chicken pox during the winter months. None developed rheumatic recrudescences.

All of the individuals were in good health on March 1.

A severe epidemic of influenza began on March 22. All but six children contracted the disease. The filterable virus responsible for this outbreak was recovered.

This agent did not activate the rheumatic process. It was followed by an outbreak of streptococcus infection and appeared to facilitate its spread.

The source of these infections was not traced. They were due to a single type of hemolytic streptococcus which was a strong toxin producer. Its cultural, biochemical and serological characteristics were different from those of the carrier strain.

Of seventeen individuals proven bacteriologically to be infected with the epidemic strain, fourteen rheumatic subjects developed acute rheumatism, two rheumatic subjects and one patient with congenital heart disease escaped.

These fourteen rheumatic attacks were accompanied by a rise in antistreptolysin titer coincident with the onset of symptoms.

In four of these attacks it was possible to exclude influenza as a causative factor.

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observations described in these papers. Whether the presence of infected tonsils served to modify the immune response and to protect D. from a recrudescence is unknown. Both patients had received 200 cc. of orange juice daily during 1935 in addition to an adequate diet.

The clinical observations, the bacteriological findings and the immunological evidence indicate that this severe outbreak of rheumatic fever was caused by *Streptococcus hemolyticus*, which appeared to be a single strain by type.

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# STUDIES ON THE IMMUNE RESPONSE OF THE RHEUMATIC SUBJECT AND ITS RELATIONSHIP TO ACTIVITY OF THE RHEUMATIC PROCESS\*

## III. OBSERVATIONS ON THE REACTIONS OF A RHEUMATIC GROUP TO AN EPIDEMIC INFECTION WITH HEMOLYTIC STREPTOCOCCUS OF A SINGLE TYPE

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PLATES 8 TO 10

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The findings presented in preceding papers indicate the importance of infection with hemolytic streptococcus in initiating rheumatic activity. It is the opinion of the authors that this is not the only factor underlying the development of the rheumatic state. However, a study of other possible contributing factors is handicapped, first, by the lack of a suitable experimental animal; second, by the absence of a laboratory test for detecting the potential rheumatic subject; third, by the uncontrollable variables of the environment in which rheumatic patients ordinarily live. This third difficulty may be partly overcome in an isolated rheumatic colony. If, under these conditions, there occurs an epidemic infection with a single effective agent, it may be possible to study the influence of other factors and their relation to the development of rheumatic activity.

The epidemic of rheumatic fever at The Pelham Home presented an opportunity for studying the responses of a rheumatic group living under similar conditions and exposed to infection with hemolytic streptococcus of a single type. The authors have paid particular attention to assessing the significance of the following factors in their relation to this outbreak: (a) housing conditions, (b) diet, (c) physical

\* The work reported in this communication was carried out under The Kellogg Foundation Fund.



status of the patient, including sexual development, (d) state of rheumatic convalescence, (e) susceptibility to infection, (f) constituents of the throat flora, (g) antistreptolysin titer previous to infection, (h) disease pattern characteristic of the subject, (i) character of streptococcus strain, (j) immune response of the host as judged by the development of antistreptolysin. The purpose of the present paper is to record the responses of this group exposed to an epidemic infection and to assess the factors significant in the different types of clinical reactions.

### *The Clinical Characteristics of the Rheumatic Outbreak*

The clinical manifestations that characterized this outbreak included almost every rheumatic phenomenon which has been seen by the authors. Disease activity was intense in eleven patients. Pain in the lumbar region was the most conspicuous symptom, and pyrexia was marked. Eight of these severe attacks were accompanied by pericarditis, the signs appearing in each instance between the 8th and 14th day of the attack. The development of anemia was striking in all eleven individuals, although with the exception of frequent mild epistaxis there was no evidence of bleeding. In five members of this group the hemoglobin readings fell from 80-90 per cent to 40-50 per cent. Three of these individuals had pleurisy and the signs of transitory pulmonary solidification. In two, there was hyperpyrexia with temperature of 106°F. All eleven had marked cardiac embarrassment. Two individuals during the early part of the disease attack developed edema of the face (see example in Figs. 2a and b) which appeared and disappeared frequently and rapidly, although nothing abnormal was found in the urine except a few erythrocytes. Rheumatic nodules were found in five of this group. The first three individuals to become ill, who had occupied adjoining beds, who, on March 30, had recovered from influenza accompanied by secondary hemolytic streptococcus infection, and who had developed rheumatic fever on April 10, awoke on May 13 and found subcutaneous nodules symmetrically distributed on each hand. When they had been examined on May 11, no nodules had been detected. Two other individuals of this group also developed nodules about 40 or 50 days after the onset of rheumatic symptoms.

The milder attacks, occurring in three individuals, were charac-

terized by less cardiac embarrassment, absence of signs of pericarditis, moderate fever, severe muscle pains, chorea, transitory cardiac arrhythmias and disappearance of all manifestations within 1 month. Seven rheumatic patients and one child with congenital heart disease escaped all rheumatic symptoms. The responses of the 24 rheumatic subjects in relation to their infections are presented in Table I.

The rapidity of the sedimentation of erythrocytes closely paralleled the clinical severity of the attacks (1-3). The hourly readings, for

TABLE I  
*The Responses of Twenty-Four Isolated Rheumatic Subjects to Epidemic Upper Respiratory Infection*

| Response of host         | No. of cases | Type of preceding respiratory infection      |                          |
|--------------------------|--------------|--|--------------------------|
|                          |              | Acute infection with hemolytic streptococcus | Following influenza      |
| Severe rheumatic attack  | 7            |  |                          |
|                          | 2            | " "  | Independent of influenza |
|                          | 2            | " "  | In absence of influenza  |
|                          | 2*           | " "  | Following influenza      |
| Mild rheumatic attack    | 2            | " "  | Independent of influenza |
| Escaped rheumatic attack | 2            | Hemolytic streptococcus present in throat    | Following influenza      |
| No rheumatic symptoms    | 4            | Hemolytic streptococcus absent from throat   | " "                      |
|                          | 3            | " "  | In absence of influenza  |

\* One atypical attack.

those with severe attacks, lay between 90 and 150 mm. and the readings for those with mild or abortive attacks ranged from 30 to 75 mm. In contrast, the patients who escaped clinical manifestations of rheumatic activity showed little increase in their sedimentation rates. The readings in these individuals were: 14, 19, 20, 30, 31 and 40. In summary, the clinical picture included almost all recognized rheumatic phenomena, especially those which characterize intense activity of this disease process. The clinical observations and blood sedimentation rates are summarized in Table II.

*Summary of the Clinical Histories of Patients at*

| Name       | Fall 1933          |        | Winter 1934                                    |               |
|------------|--------------------|--------|--|---------------|
|            | Clinical condition | E.S.R. | Clinical condition                             | E.S.R.        |
| Acconero   | Subsiding chorea   |        | Good health                                    | 10            |
| Allen, G.  | Subsiding carditis | 16     | Good health                                    | 7             |
|            | Subsiding chorea   |        |  |               |
| Allen, H.  | Subsiding carditis |        | Good health                                    | 15            |
|            | "Colds"            |        |  |               |
| Bourie     | In N. Y. C. Sub-   |        | In N. Y. C. Good health.                       | Admitted to   |
|            | siding carditis    |        |  | pharyng       |
| Campbell   | In N. Y. C. Good   |        | In N. Y. C. Acute rheumatic attack. Resp.      | 130           |
|            | health             |        | hist. unknown                                  | Admitted to   |
| Carroll    | Good health        | 4      | "Colds," vague mild symptoms                   | 6             |
| Cleary     | Good health        | 13     | "Colds"  | 6             |
| Curnan     | Good health        | 6      | "Colds"  | 6             |
| Davis      | In N. Y. C. Good   |        | In N. Y. C. Good health                        | Admitted to   |
|            | health             |        |  | pharyng       |
| Delahanty  | Good health        |        | In N. Y. C. Rheumatic attack                   | 55            |
| Doran      | Good health        | 10     | Good health, discharged P.H. January           | Admitted to   |
| Earls      | Good health        | 6      | Chicken pox                                    | 7             |
| Fay        | "Colds"            | 17     | "Colds"  | 13            |
|            |                    |        |  | attack N.Y.   |
| Ferrara    | Good health        | 7      | Good health                                    | R.I. Mar. 21  |
| Gamel      | Good health        | 10     | Good health                                    | R. I. Mar. 21 |
| Graback    | In N. Y. C. Sub-   |        | Subsiding rheumatism. Admitted Mar. 14 P.H.    | 25            |
|            | siding attack      |        |  | R.I. Apr. 15  |
| Goch       | Good health        | 15     | "Cold"   | 18            |
|            |                    |        |  | R.I. Apr. 15  |
| Gross      | In N. Y. C. Sub-   |        | Admitted P.H. Feb. 25. Subsiding carditis      | 14            |
|            | siding carditis    |        |  | R.I. May 19   |
| Grossman   | In N. Y. C. Good   |        | In N. Y. C. Severe rheumatic attack. Resp.     | 100           |
|            | health             |        | hist. unknown                                  | Admitted to   |
| Gunning    | Good health        | 22     | "Colds," mild, vague symptoms                  | 6             |
|            |                    |        |  | R.I. Mar. 22  |
| Hudson     | In N. Y. C. Good   |        | In N. Y. C. Rheumatic attack. Resp. hist.      | 115           |
|            | health             |        | unknown  | Admitted to   |
| Hallahan   | "Cold"             | 10     | "Cold"   | 10            |
| Kienzle    | Good health        | 23     | "Colds," vague rheumatic symptoms              | 15            |
| Kiernan    | Good health        |        | Good health                                    | 8             |
| Libera     | Good health        |        | Good health                                    | 12            |
| Marten     | Good health        | 13     | Chicken pox                                    | 21            |
| McMahon    | Good health        | 4      | Good health                                    | 15            |
| Patterson  | Subsiding carditis | 30     | Good health                                    | 5             |
| Raimonde   | Subsiding carditis | 24     | "Cold"   | 33            |
|            |                    |        |  | attack N.Y.   |
| Ryan       | Good health        | 2      | "Cold"   | 12            |
| Stravelli  | Good health        | 18     | Good health                                    | 11            |
| Sucich     | Good health        |        | Admitted P.H. Mar. 1. Congenital heart disease | 8             |
| Torres     | "Cold"             | 5      | Chicken pox                                    | 10            |
| Westwater  | Subsiding carditis | 55     | Symptom-free                                   | 10            |
| Williamson | Good health        | 12     | "Cold"   | 4             |
|            |                    |        |  | R.I. Apr. 15  |

E.S.R. = erythrocyte sedimentation rate. R.I. = respiratory infection, details in text. P.H. = physical hospital



*1. The Clinical Histories of Patients Developing Severe Rheumatic Attacks at The Pelham Home.*—The first group of patients consists of eleven individuals who were symptom-free in March, 1934, and who developed severe rheumatic recrudescences following hemolytic streptococcus infection with organisms of a single type. The following clinical histories and autopsy findings illustrate the character of these attacks.

Patient Westwater, a 13 year old girl, born in England of British parents, was admitted to The Pelham Home in November, 1933. She was convalescing from a recent rheumatic attack and had advanced mitral stenosis. The disease picture began with a severe attack of polyarthritis in Birmingham, England, at the age of 7. At that time the patient's sister contracted this disease, and the mother died of it. Shortly after arriving in New York she developed another rheumatic attack and was admitted to St. Vincent's Hospital. The manifestations included carditis, muscle pains, epistaxis, polyarthritis, purpura, pallor, wasting and subcutaneous nodules.

Her course at The Pelham Home was one of slow improvement until the winter of 1934 when her progress became rapid. On Mar. 20 she was in good condition when she contracted influenza which was complicated by pharyngitis. She was then symptom-free until Apr. 7 when she complained of painful joints. Within 2 days this patient developed a severe rheumatic attack. Pain in the lumbar region was especially marked; epistaxis, muscle pains, polyarthritis were severe; the body temperature was elevated to 102°. The symptoms increased in intensity; pallor became marked; the signs of cardiac insufficiency followed. The liver became markedly enlarged during the 3rd week; dyspnea and orthopnea increased in severity. On May 13 rheumatic nodules were detected. The hemoglobin fell to 60 per cent. Nausea and vomiting became uncontrollable, and the patient was transferred to the Presbyterian Hospital, where she was found to have marked electrocardiographic changes. Activity of the disease process continued throughout the summer months.

*Bacteriological Findings.*—The throat flora was free of hemolytic streptococcus until Mar. 26 when, at the time of pharyngitis, the epidemic type of this organism (Strain EpEW) appeared in moderate numbers.

*Impression.*—A rheumatic subject, while convalescent from a recent rheumatic attack, contracted influenza. This infection was complicated by hemolytic streptococcus pharyngitis and was followed by a severe rheumatic recrudescence.

Patient Raimonde, a girl of 7, born in New York City of Italian parents, was admitted to The Pelham Home in November, 1933. At that time she was re-

covering from a rheumatic attack with auscultatory signs of involvement of the mitral valve. The disease picture had been vague in the past. She was not known to have rheumatic disease when she was admitted to the Babies Hospital in October, 1933, because of abdominal pain. While under observation there she developed frequent, severe epistaxis and pyrexia up to 105°, tachycardia, secondary anemia and a loud systolic apical murmur. Recovery was rapid.

Her course at The Pelham Home was one of steady improvement throughout the winter and spring months. On Mar. 26 she contracted influenza and was prostrated for 3 days. Recovery seemed complete, and she was attending school when, on May 3, she had a severe pharyngitis, which persisted for 4 days. Although she was symptom-free, bed rest was maintained during the following nine days. On May 16 her body temperature rose to 102°. This was followed by a severe rheumatic attack characterized by pain in the back, nausea, vomiting, muscle pains, joint pains, marked dyspnea and orthopnea. A pericardial friction rub was detected on the 10th day, and she was transferred to New York Post Graduate Hospital. There her condition became worse. Intense pallor developed. 10 days later she was critically ill with hyperpyrexia, temperature 106.6°. Symptoms and signs of myocardial failure were more marked, and secondary anemia became striking. She remained dangerously ill during the summer months when she was taken home from the hospital during continued rheumatic activity, against the advice of her physician.

*Bacteriological Findings.*—The throat flora was free of hemolytic streptococcus until May 3 when, at the time of pharyngitis, the epidemic type of this organism (Strain EpER) appeared in moderate numbers.

*Impression.*—A rheumatic subject who had recently experienced an attack of carditis contracted influenza without sequelae. Five weeks later she became infected by hemolytic streptococcus and developed a fulminating and perhaps fatal rheumatic recrudescence.

Patient Fay, an 11 year old girl born in New York City of Irish parents, was admitted to The Pelham Home in March, 1933. At that time she was recovering from a rheumatic recrudescence and had the signs of mitral stenosis. The disease picture had included many previous attacks of rheumatic fever with a great variety of manifestations. She had polyarthritis at 6 years, severe carditis at 8 years, polyarthritis at 9 years, severe epistaxis, polyarthritis and pericarditis at 10 years of age.

Her course at The Pelham Home was not marked by striking improvement until November, 1933. During the early part of 1934 she was in excellent condition. On Mar. 20 she contracted influenza and on Apr. 22 acute tonsillitis. Recovery was rapid, and she remained symptom-free until May 17. On that day the rheumatic attack began with pyrexia, polyarthritis and muscle pains. Active carditis continued throughout the summer months.

*Bacteriological Findings.*—The throat flora contained the carrier strain during

January and February, but was free of hemolytic streptococcus during March and most of April. This organism was not recovered at the time of influenza; however, with the onset of tonsillitis, the epidemic type of this organism (Strain EpEF) appeared in predominance, remained prominent for 3 weeks and then disappeared from the throat cultures.

*Impression.*—A rheumatic subject contracted influenza and escaped a rheumatic recrudescence. Acute tonsillitis 1 month later was followed after an interval of 14 days by a severe rheumatic attack from which she is slowly recovering.

Patient Hallahan, a 14 year old girl born in New York City of Irish parentage, was admitted to The Pelham Home in December, 1929, with the physical signs of mitral stenosis. The disease picture had been characterized by growing pains, mild joint pains and two mild attacks of rheumatism with slight pyrexia, precordial pain, dyspnea and muscle pains.

Her course at The Pelham Home was in general satisfactory. She had a mild rheumatic attack during the winter of 1932. In 1933 and at the beginning of the present study she was in excellent health. On Mar. 24, 1934, she contracted influenza complicated by pharyngitis. On Apr. 7, 12 days later, a fulminating rheumatic attack began with epistaxis and a temperature of 104.6°F. This was followed 10 days later by the onset of auricular fibrillation. Severe pain in the back, mild polyarthrititis, muscle pains, nausea and vomiting, cephalalgia, increasing dyspnea and orthopnea, intense pallor and anxiety comprised the clinical picture. Rheumatic nodules appeared on May 13 at a time when the intensity of the disease appeared to be subsiding. Another cycle was accompanied by such severe and prolonged vomiting that it was necessary to transfer the patient to the Presbyterian Hospital for treatment. There it was found that she had developed a secondary anemia and electrocardiographic changes, auricular fibrillation, marked right preponderance and inversion of T wave in the second lead. Her condition was improving steadily when on May 30, 1 week after admission, she complained of severe abdominal pain, became apprehensive and died suddenly 12 hours later.

*Bacteriological Findings.*—These findings may be summarized as follows: The throat flora was free of hemolytic streptococcus during 1933 and 1934 until she contracted influenza at the end of March. This infection was complicated by pharyngitis, with hemolytic streptococcus of the epidemic type (Strain EpEH) in preponderance.

### *Gross Changes*

The autopsy, performed by Dr. Dorothy Andersen, is summarized briefly. The gross findings were striking and almost identical with those of a patient previously reported (4).<sup>1</sup> There was a moderate amount of blood-tinged fluid in

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<sup>1</sup> Coburn (4), Case E. B., page 936.

the body cavities. The heart, and especially the right ventricle, was markedly enlarged. There was roughening of the surface over the auricles and strands of fibrin were found between the auricular appendage and aorta. Fresh rheumatic vegetations were present on all of the heart valves. The striking pathological change was multiple hemorrhages in the fatty tissues. The condition in the abdominal cavity resembled an extensive purpura. The omentum and mesentery were mottled red, studded with hemorrhages, varying in size from petechial to 6 mm. These were especially marked in the mesocolon. (See Fig. 1.) Likewise the surface of the heart was remarkable in its appearance because of the great number of petechial hemorrhages. They were numerous around the vessels, in the fat and confluent in the auriculoventricular groove. Several of the tracheo-bronchial lymph nodes were large, edematous, deep purple in color, surrounded by hemorrhage.

### *Microscopic Changes*

The microscopic studies showed two types of changes, those of specific histological character and those associated with the multiple hemorrhages. Sections from each of the valves showed the characteristic picture of acute rheumatic endocarditis. In a clot from the pericardium there were strands of material resembling fibrin and cells resembling Aschoff cells. The changes in the heart muscle were widespread. Aschoff bodies were numerous and large. In some areas fragmented collagen alone was seen; in others there were just Aschoff cells; in the neighborhood of some of the large Aschoff bodies the muscle fibers were destroyed. There was a typical auriculitis. The walls of many small vessels were thickened. The changes in a branch of the coronary artery were marked. The intima was thick, irregular and contained many large nuclei resembling those of Aschoff cells. At one point a large collection of these cells was oriented at right angles to the surface. There was also thickening of the adventitia of this vessel and destruction of the muscularis.

The widespread distribution of hemorrhages was striking. Many large hemorrhages were present in the epicardial fat. Collections of polymorphonuclear leucocytes were seen. A similar picture was seen in the adventitia of the aorta, where in a few instances the cells surrounded small areas of pale, somewhat eosinophilic homogeneous material. The alveoli of large areas of the lungs were filled with fresh red blood cells. There were hemorrhages into the liver columns, in the kidney pyramids and in the bone marrow. The most widespread of all were in the omentum. Here, also, there were accumulations of polymorphonuclear leucocytes and sometimes a small necrotic area in the center of the leucocytes.

The tonsillar tag contained exudate and Gram-positive cocci. (See Fig. 4.) The bronchial lymph nodes were large, congested, edematous, studded with hemorrhages containing a few polymorphonuclear leucocytes. (See Fig. 5.) Gram stains of fluid from the center of the glands contained a large number of Gram-positive cocci in chains with many degenerating forms, which did not grow on blood agar under aerobic or anaerobic conditions.



*Impression.*—A rheumatic subject in excellent health, under close observation for 5 years, contracted influenza which was complicated by pharyngitis with hemolytic streptococcus. After being symptom-free for 10 days the patient developed a fulminating attack. While apparently recovering, she died with severe abdominal pain, which necropsy showed to be due to hemorrhage.

Patient Patterson, a 7 year old girl born in New York City of Irish parents, was admitted to The Pelham Home on Sept. 23, 1933, while recovering from an attack of rheumatic fever with carditis. The disease picture had appeared in the spring months of 1933 and was characterized by fatiguability, growing pains, listlessness, mild fever, abdominal pain and, on physical examination, tachycardia, cardiac hypertrophy with a mitral bulge and the auscultatory signs of mitral stenosis.

Her course at The Pelham Home was one of steady progress until May 15, when she contracted severe pharyngitis. On June 8 a rheumatic attack began with fever, generalized urticaria and muscle pains. She was transferred to the Babies Hospital 2 days later in good condition with only mild pyrexia. The intensity of the disease increased each day. Pallor became marked; then drowsiness; on the 10th day a pericardial friction rub was detected; suddenly extreme pulmonary embarrassment appeared overwhelming. She was placed in an oxygen tent with moderate temporary relief of the extreme cyanosis. Death occurred on June 22, at the end of the 2nd week of the attack.

*Laboratory Findings.*—Hemolytic streptococcus in small numbers had been present in the throat flora during the fall and early winter months (Strain NEP) but absent during April and May until the epidemic type (Strain EpEP) appeared in large numbers at the onset of pharyngitis. The hemoglobin fell 10 per cent and the red blood count 1 million during the first 5 days in the hospital.

### *Gross Changes*

The autopsy, performed by Dr. Beryl Paige, is summarized briefly. The gross findings were characteristic of a number of patients dying at the end of the first cycle of a rheumatic attack. The heart was large, and its outer surface was roughened by extensive fibrinous exudate and delicate fibrous adhesions. The mitral valve was thickened and showed rows of fresh verrucae along the leaflets. The chordae tendineae were thickened, shortened and pearly gray. There were tiny hemorrhages in the mesentery, along its attachment to the terminal portion of the ileum, about the rectum and in the retroperitoneal tissue. The lungs were heavy. There was a fine deposition of fibrin on the pleural surface. All lobes were deep beefy red, firm, moist and on section slightly granular, containing sharply demarcated pale pink areas.

*Microscopic Changes*

The microscopic studies showed specific lesions in the mitral valve, left auricle, left ventricle and pericardium. There was a central necrosis about the efferent veins of the liver lobules and accumulations of polymorphonuclear leucocytes. (Fig. 3.) No histological signs of passive congestion were found in other organs. The histological changes in the lungs were extensive and striking. (Fig. 6.) On the pleural surface there was a layer of fibrin. The alveoli were filled with coagulated fluid. Some were lined with a wide, eosinophilic hyaline membrane and many contained polymorphonuclear leucocytes. In many of the lumina of the capillaries in the alveolar walls were heavy masses of hyaline material which gave the staining reaction of fibrin. In these regions many of the nuclei in the alveolar walls were pyknotic, fragmented or distorted into bizarre forms. The interlobar septa were edematous, and scattered leucocytes were present in the interstices of the areolar tissue. The lymphatics were dilated. The sinuses of the bronchial lymph gland were dilated, filled with polymorphonuclear leucocytes, red blood cells and large mononuclear cells showing erythrophagocytosis.

*Impression.*—This child was a rheumatic subject who had recently experienced her first attack. She was in good condition, escaped influenza at The Pelham Home but later contracted a throat infection with the highly effective strain of hemolytic streptococcus associated with this epidemic. The rheumatic recrudescence appeared late, nearly 4 weeks after infection and 13 days after the rise in antistreptolysin titer. It was characterized by those manifestations which are associated with intense activity of the rheumatic process and ended fatally with pulmonary solidification.

These two individuals died during activity of the rheumatic process. Neither had signs of chronic passive congestion nor symptoms of cardiac insufficiency. Death occurred in the first patient during the third cycle, and the anatomical findings indicated that the sudden fatality was associated with hemorrhage. Bleeding occurred mostly in fat and except for the lung did not involve parenchymal tissues. In the second patient, death resulted from the hemorrhagic consolidation of almost the entire lung tissue. Neither arterioles nor capillaries showed inflammation or other changes. Lesions with specific histological character were present in great numbers through the hearts of both individuals. The anatomical findings indicated the intensity of the disease process and suggested the type of tissue changes which were presumably taking place in the organs of the nine other patients with severe recrudescences.

The above clinical histories are typical of the eleven individuals who developed severe rheumatic attacks during the epidemic at The Pelham Home. The patients responded to infection with striking uniformity. All had severe carditis; eight developed signs of pericarditis between the 10th and 14th day of the attack; in three children subcutaneous nodules appeared symmetrically distributed over both hands on the 48th day after infection. Some of the conditions underlying the development of these rheumatic attacks were common to the group; others differed from one individual to another.

(a) *Housing*.—All of these patients had resided at The Pelham Home, Pelham Manor, New York, for at least 6 months previous to the epidemic infection. They had been given the best living conditions possible in the suburban environment of New York. Their home was clean, dry, well lighted and well ventilated. An even indoor temperature, proper clothing, freedom from exposure to inclement weather and adequate rest were maintained throughout the period of observation. All of the children had attended school in one room of the building where the transmission of respiratory infection may have been facilitated. They were all subject to the spring climate of the North Temperate Zone.

(b) *Diet*.—The entire group of patients were on a regular diet. This included three large meals and extra milk. The meals were well balanced, and the children were required to eat all of their food. Oranges and fresh vegetables were served each day. The undernourished children received cod liver oil. All of the children gained weight and most of them were developing a good state of nutrition at the time of the rheumatic outbreak.

(c) *Physical Status*.—No two individuals were in the same physical condition. Patients Hallahan and Westwater had passed puberty. Menstruation had not begun in the other children. Five had experienced many attacks of carditis and had advanced heart disease (Westwater, Gamel, Goch, H. Allen, Williamson). Four individuals had only the early signs of cardiac involvement (Raimonde, Patterson, Gunning and Earls). Two other patients (Fay and Hallahan) also had signs of moderate heart involvement; but their general robust appearance was strikingly different from the group of five with advanced heart disease. Hallahan, who had been at The Pelham Home

for 5 years, appeared to be the one patient in excellent physical condition.

(d) *State of Convalescence*.—Nine of the patients mentioned above were in a convalescent stage, recovering from attacks in 1933. Fay and Hallahan had been symptom-free for more than 1 year.

(e) *Susceptibility to Infection*.—All of the group had contracted the common cold at some time during their stay at The Pelham Home. None of them seemed unusually susceptible or immune to upper respiratory tract infections.

(f) *Character of Throat Flora*.—The throat flora of the group had in most instances been free of pathogenic organisms before the epidemic. Prior to March only two patients carried hemolytic streptococcus in the throat flora. In these individuals, Fay and Patterson, the carrier strain (NEP) was present in moderate numbers during the winter months, but disappeared about 3 weeks before the epidemic.

(g) *Level of Antistreptolysin Titer Previous to Infection*.—The anti-streptolysin titers of these eleven patients were approaching natural level at the time of the epidemic. Titers were moderately elevated in two individuals. None had high titers prior to the epidemic. See Text-figs. 6 and 7, pages 153 and 154, Paper II.

(h) *The Disease Pattern Characteristic of the Subject*.—All of these individuals had in the past experienced frank attacks with heart involvement. They seemed highly susceptible to rheumatic carditis. The recrudescences at The Pelham Home were of a similar character but more intense than any of their previous attacks.

(i) *Type of Infectious Agent*.—All of these patients appeared to be infected with a single type of hemolytic streptococcus (this was not proven in the case of Gamel) which was a strong toxin producer and highly pathogenic for mice. In seven of the group the streptococcus infections complicated influenza. In Raimonde and Fay the streptococcus infections occurred independently of influenza. Goch and Patterson escaped influenza entirely. In all eleven patients the organism was highly effective and strikingly different from the non-effective strain which had been prevalent in the throat flora during the winter months.

(j) *Immune Response of the Host*.—All eleven patients developed a sharp rise in antistreptolysin titer, coincident with the onset of the

rheumatic attack. With the exception of Patterson who died early in the attack, the titers reached unusually high levels. See Text-figs. 6 and 7, pages 153 and 154, Paper II.

2. *Summarized Clinical Histories of Patients with Mild Rheumatic Attacks Following Hemolytic Streptococcus Infection.*—Three children became infected with the epidemic type of hemolytic streptococcus and developed rheumatic symptoms which, although definite, were mild. Patient Grabeck, with mitral stenosis and a history of vague rheumatic symptoms, escaped influenza but had a mild pharyngitis with hemolytic streptococcus of the epidemic type during the 2nd week in April. This was followed after 10 days by a rise in the blood sedimentation rate to 75 mm., and evidence of rheumatic activity. All symptoms and signs disappeared in 2 weeks; but the sedimentation rate remained elevated for 6 weeks. Patient Gross, a rheumatic subject who had previously had chorea and carditis with transitory auricular fibrillation, escaped all symptoms of respiratory infection. However, the epidemic type of hemolytic streptococcus appeared in the throat flora during the 1st week of May. This was followed 2 weeks later by chorea, a moderate rise in the blood sedimentation rate and paroxysmal auricular fibrillation, with symptoms of cardiac insufficiency. Patient Torres, a rheumatic subject who in the past had experienced mild polyarthritides and vague cardiac symptoms, contracted influenza on Mar. 23. Hemolytic streptococcus appeared in the throat flora a few days later. On Apr. 9 she developed mild rheumatic activity, with symptoms persisting for 3 weeks. The blood sedimentation rate rose only moderately during the period of symptoms. The antistreptolysin titer curves of these three patients are presented in Text-fig. 5, page 152, Paper II.

Unlike the first eleven patients, these three children had only mild recrudescences. There was no detectable difference between the two groups in respect to housing, diet, physical status, state of convalescence, susceptibility to infection, character of the throat flora prior to infection or type of infectious agent. These three children were infected with the epidemic type of hemolytic streptococcus, which in one instance complicated influenza. There were two detectable differences, the character of their previous rheumatic attacks, and the intensity of the immune response. In the past none of these three

children had had frank attacks but had either experienced mild rheumatic symptoms or had developed the early signs of heart disease insidiously. Although they were infected at the same time with the same type of agent as the preceding group, they developed only a moderate elevation of antistreptolysin level.

*3. Clinical Histories of Patients Escaping Rheumatic Attacks at The Pelham Home.*—Four children experienced clinical infection with hemolytic streptococcus of this epidemic type, yet failed to develop rheumatic manifestations. Like the preceding group none had experienced severe rheumatic attacks in the past. One patient had only congenital heart disease; one was an atypical rheumatic subject; two had had many mild rheumatic manifestations but little cardiac damage.

*Non-Rheumatic Subject Who Had No Rheumatic Symptoms Following Hemolytic Streptococcus Infection.*—Patient Such was a child with congenital heart disease who had at no time manifested any rheumatic symptoms. She escaped influenza at The Pelham Home; however, on May 5 she contracted pharyngitis with the epidemic type of hemolytic streptococcus (Strain EpS). This infection was accompanied by a rise in the blood sedimentation rate to 40 mm. Recovery was rapid, and she developed no evidence of rheumatic disease, although the antistreptolysin titer rose at the time that the rheumatic recrudescence ordinarily occurs.

*An Unusual Rheumatic Subject with an Atypical Response to Infection with Hemolytic Streptococcus.*—Patient McMahon had experienced many attacks of mild polyarthritis without ever developing carditis. She carried hemolytic streptococcus in the throat flora during the winter and spring months. On April 11 she contracted influenza, followed by severe bronchitis. The epidemic type of hemolytic streptococcus appeared in the throat cultures at that time along with the carrier strain. On April 21 she developed severe polyarthritis. For a week the temperature exceeded 104° almost every day. Exquisite tenderness of the joints and muscles and marked pyrexia were not affected by large doses of sodium salicylate. The sedimentation rate during this acute illness remained low. However, all symptoms disappeared in 2 weeks and the heart appeared perfectly normal at the end of this time. She experienced no cardiac symptoms on resuming

unrestricted physical activity. It has been difficult to determine whether this child is really a rheumatic subject. Her clinical response has always been unusual, resembling that of an elderly person. Her case is classed as mild rheumatism in Table I, page 161.

*Rheumatic Subjects Who Escaped Recrudescences Following Hemolytic Streptococcus Infection.*—Patient Acconero, a child with mitral stenosis, was known to be a rheumatic subject. She had harbored a carrier strain of hemolytic streptococcus in her throat during the entire winter. On April 13 she contracted a respiratory infection, and the epidemic type of hemolytic streptococcus appeared in her throat flora. This was followed by a rise in the blood sedimentation rate to 65 mm. 2 weeks later the sedimentation rate returned to a normal level. This child, who in the past had had frequent mild rheumatic symptoms, did not manifest definite evidence of rheumatic activity. Patient Stravelli, a child with mitral stenosis, was also known to be a rheumatic subject. Likewise, she had carried hemolytic streptococcus in the throat flora during the late winter and spring months. She experienced a respiratory infection on April 8. Following this the throat flora contained both strains of hemolytic streptococcus, the epidemic strain and the one that had been carried. During the 1st week in May the blood sedimentation rate rose to 66 mm., returning to a normal level 2 weeks later. Like the first child, this patient did not manifest definite evidence of activation of the rheumatic process.

The first two patients of this group were probably constitutionally different from rheumatic subjects. The last two patients in the past had behaved clinically like the three individuals who developed mild recrudescences. Although infected with hemolytic streptococcus of the epidemic type, these two rheumatic subjects showed little or no change in antistreptolysin titer and escaped recrudescences. (See Text-fig. 4, page 151, Paper II.)

*4. Clinical Histories of Patients Who Failed to Contract the Epidemic Hemolytic Streptococcus Infection.*—Three children escaped all respiratory infection and four children contracted only influenza during the epidemic at The Pelham Home. None developed any rheumatic manifestations.

*Patients Who Escaped Both Influenza and Also Secondary Infection with Hemolytic Streptococcus.*—Three children contracted no infection

whatever during the epidemic. Two had been at The Pelham Home for 2 years and were known to be highly susceptible rheumatic subjects. During the period of subsiding rheumatism in 1932, their titers had been 500 units (Kienzle) and 333 units (Curnan). Kienzle experienced a mild recrudescence about 1 month before the epidemic; Curnan had been entirely symptom-free for more than 1 year. Neither developed any evidence of rheumatic activity during the epidemic of 1934, and there was no significant increase of antibody. The third patient, Hudson, was admitted to The Pelham Home 3 months before the epidemic with subsiding rheumatism. She escaped influenza and did not develop symptoms or signs of pharyngitis although the epidemic strain of hemolytic streptococcus appeared in her throat flora. The blood sedimentation rate fell to normal and the antistreptolysin titer remained constantly at a high level.

*Patients Who Contracted Influenza Yet Escaped Secondary Infection with Hemolytic Streptococcus.*—Four children contracted influenza during the first part of April, but appeared to escape streptococcus infection. Libera had been clinically quiescent for one year, and had maintained an antistreptolysin titer of 143 units. Kiernan had had chorea during the preceding summer. Carroll had been admitted to The Pelham Home in 1931 with acute rheumatism and had experienced smouldering activity in 1932 with a titer of 1,000 units which fell to 250 units during 1933. She was in good health at the beginning of spring in 1934 with a titer of 125. G. Allen was admitted to The Pelham Home in the summer of 1933 with active rheumatism, running a constant titer of 500 units. She regained good health during the fall and the titer fell to 143. None of these individuals developed any clinical evidence of rheumatic activity following the epidemic in the spring of 1934.

These seven patients were rheumatic subjects and in the past had been susceptible to infection. None of them contracted hemolytic streptococcus infection during The Pelham Home epidemic although four had influenza. None developed a high antistreptolysin titer and none had rheumatic recrudescences. These seven individuals differed from the first group who contracted infections in only two recognizable respects. All who escaped infection carried a non-effective strain of hemolytic streptococcus in the throat flora and with



one exception their antistreptolysin titers had not returned to a natural level (5) at the onset of the epidemic. (See Text-figs. 2 and 3, page 151, Paper II.)

#### DISCUSSION

This epidemic made it possible to observe the clinical reactions and anatomical changes in an isolated group of rheumatic subjects who were infected with hemolytic streptococcus of a single type. The relative significance of infection, of environmental factors and of the state of the subject, ordinarily difficult to assess, can be estimated in this group.

It was definite that an adequate diet, a good state of nutrition, an even indoor temperature, proper clothing, freedom from exposure and sufficient rest did not prevent severe rheumatic disease. Two of the intense attacks occurred in children who had been at The Pelham Home for a long time and had regained excellent health. Likewise, prolonged freedom from rheumatic activity seemed of no protective value, as severe attacks occurred both in children who had recently recovered and in those who had been quiescent for more than a year. In this particular group, age and the state of sexual development were entirely indifferent. Two of the severe attacks, including one fatality, occurred in children who had passed puberty. Furthermore, no evidence was found for the existence of a refractory state during quiescence of the rheumatic process. All but two of the rheumatic subjects infected with the epidemic type of hemolytic streptococcus developed acute rheumatism irrespective of their degree of convalescence. Those individuals who escaped infection with the epidemic strain were, with one exception, carriers of hemolytic streptococcus and had at that time a moderately elevated antistreptolysin titer. It is not known whether this is significant, and if so, whether it is pertinent to those five patients admitted to The Pelham Home during the epidemic who had high antistreptolysin titers (Text-fig. 1, page 149, Paper II), hemolytic streptococcus in the throat flora and signs of active rheumatism. The purpose of this study was to determine the conditions responsible for this outbreak of acute rheumatism in fourteen children, and to explain the failure of nine other susceptible subjects to develop recrudescences. The occurrence of the outbreak seemed to be asso-

ciated with a highly effective agent, infecting susceptible subjects and initiating an intense immune response. The failure of seven individuals to contract infection with hemolytic streptococcus explains their escaping recrudescences. It is difficult, however, to understand the two susceptible patients who appeared to be infected but remained symptom-free. The only distinguishing feature of these two infections was that the antistreptolysin titer did not rise. The development of a rheumatic recrudescence appears to depend not only upon the character of the infectious agent, but also upon the immune response of the host.

#### SUMMARY

This study of an isolated colony showed that of seven children who escaped the epidemic streptococcus infection none developed rheumatic symptoms; and that of seventeen children who contracted the epidemic streptococcus infection, fourteen developed acute rheumatism and three showed no recognizable rheumatic manifestations.

The seven children who failed to contract infection with *Streptococcus hemolyticus* showed clearly that susceptible individuals may live in close association with an epidemic of acute rheumatism, develop no rise in antistreptolysin titer and maintain excellent health.

The patient with congenital heart disease demonstrated that a non-rheumatic subject may be infected with a highly effective strain of hemolytic streptococcus, and develop a typical antibody response, yet escape all rheumatic manifestations.

The two patients who, although infected with the epidemic strain, failed to show any antibody response, also failed to develop rheumatic recrudescences.

Environmental, dietary, age and the other factors investigated did not appear to be significant in this outbreak of acute rheumatism.

Three factors appeared to determine the development of the fourteen recrudescences: (1) infection with a highly effective agent; (2) the disease pattern, peculiar to each rheumatic subject; (3) the intensity of the immune response of the patient as indicated by the rise in antistreptolysin titer.

To the three nurses, Mary Kelly, Lucille Miller, and Ruth Colby, the care of the patients was entrusted. Dr. Lucille Moore and Miss

Eleanor M. Kapp assisted the authors in many ways. The advice of Dr. Alphonse R. Dochez was invaluable. With the help of these associates and with the assistance of The Kellogg Foundation the authors have been able to conduct the present studies.

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#### EXPLANATION OF PLATES

##### PLATE 8

FIG. 1. Patient Hallahan. Loop of colon. Mesocolon was studded with hemorrhages, which were confined to fatty tissues.

##### PLATE 9

FIG. 2. Patient H. Allen. (a) Normal appearance. (b) Marked transitory edema of face appearing at onset of rheumatic activity and not related to cardiac failure.

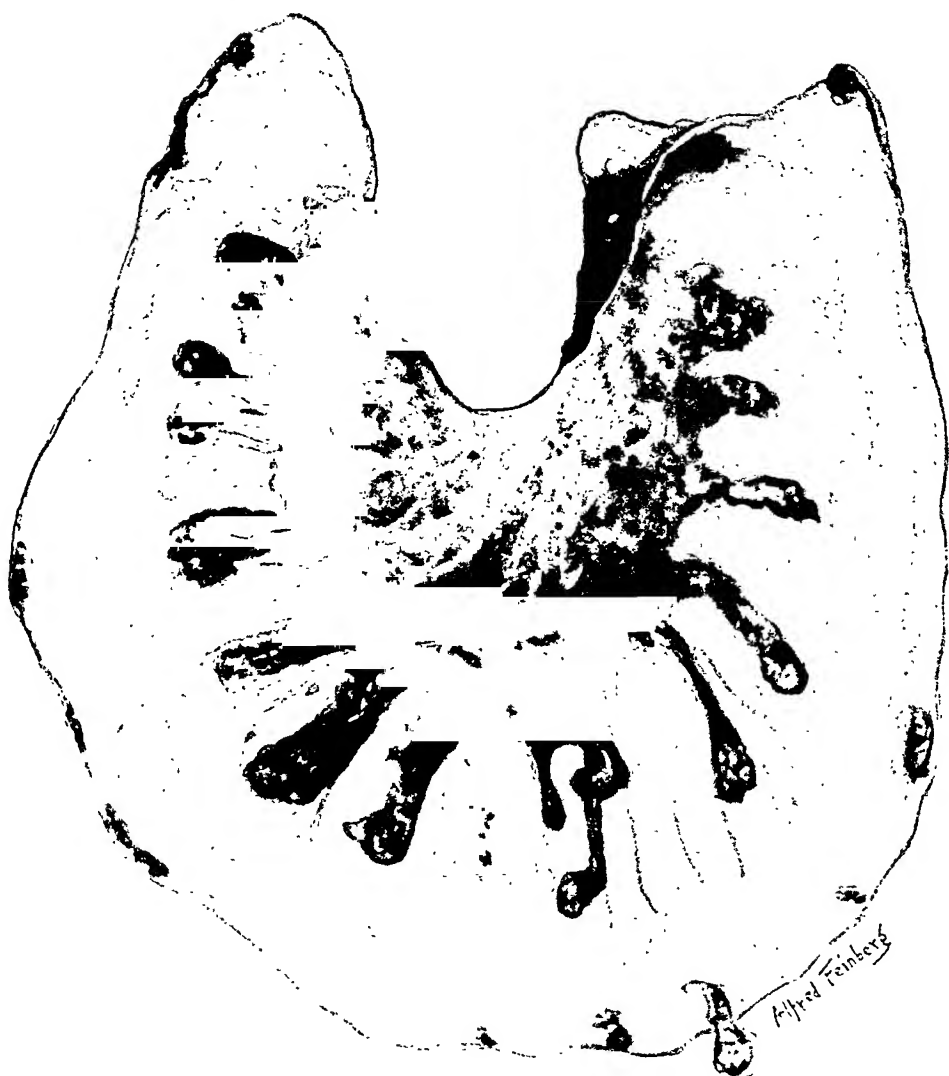
FIG. 3. Patient Patterson. Liver lobule showing central necrosis and polymorphonuclear infiltration about efferent vein.

##### PLATE 10

FIG. 4. Patient Hallahan. Remnant of tonsillar tissue under oil immersion, showing intense polymorphonuclear infiltration about Gram-positive cocci in chains.

FIG. 5. Patient Hallahan. Tracheobronchial lymph node showing capillary congestion with hemorrhage, and extensive edema and hemorrhage of the capsule.

FIG. 6. Patient Patterson. Lung under high magnification. Alveoli filled with coagulum containing polymorphonuclear leucocytes and clusters of large mononuclear cells. Alveolar walls lined with eosinophilic, hyaline membrane. Alveolar capillaries filled with heavy masses of fibrin.



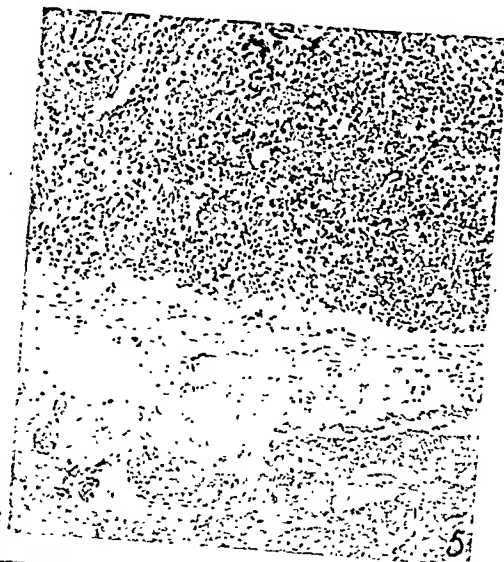
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(Coburn and Pauli: Immune response of the rheumatic subject. III)

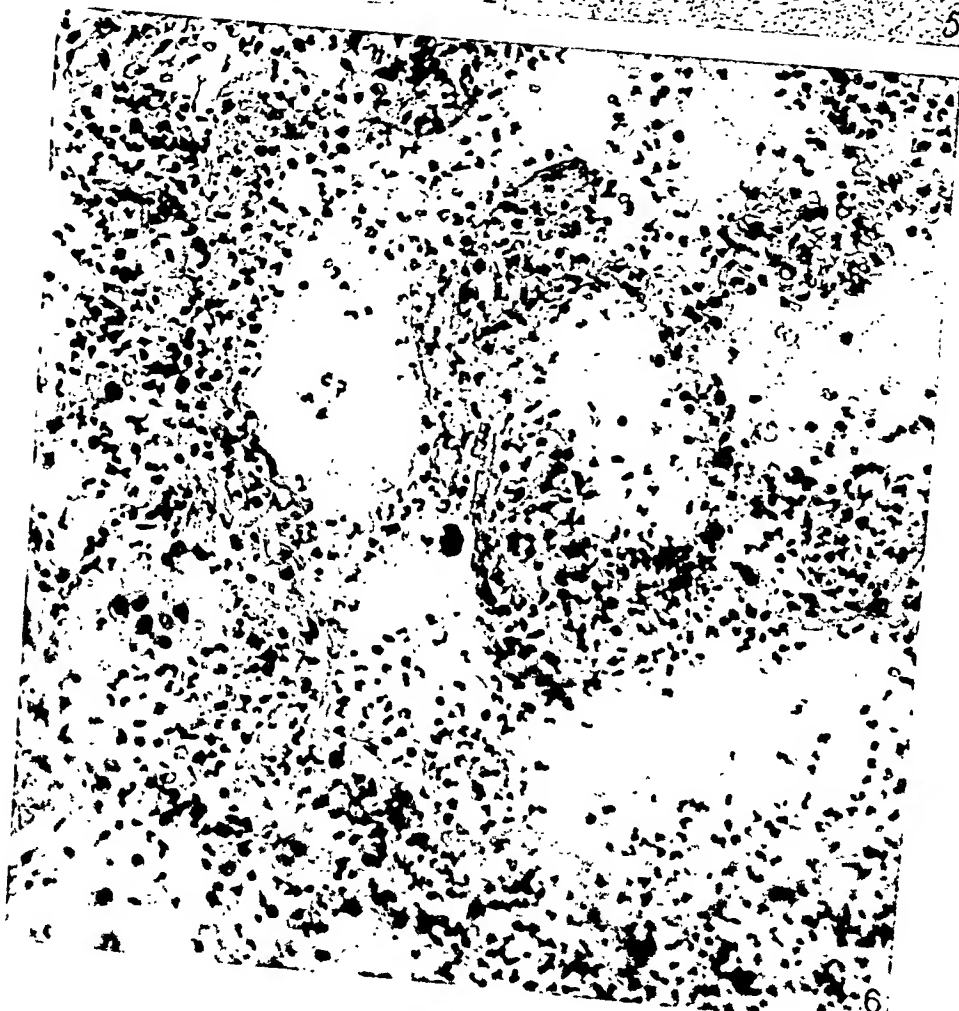




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# THE PURIFICATION OF THE ANTIBODIES IN TYPE I ANTI-PNEUMOCOCCUS SERUM, AND THE CHEMICAL NATURE OF THE TYPE-SPECIFIC PRECIPITIN REACTION

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Some years ago it was shown by Avery (1) that the protective substances of antipneumococcus horse serum are intimately associated with the globulins. By means of fractionation with ammonium sulfate it was demonstrated that the antibodies are distributed among the eu- and pseudoglobulin constituents. The immune bodies were likewise found to be partially precipitated when antiserum is saturated with sodium chloride, or when the electrolytes, normally present in the serum, are removed by dialysis.

Later methods for the isolation of pneumococcus antibodies were directed toward the dissociations of the immune precipitate obtained by the absorption of antiserum with pneumococci of the homologous type, or with aqueous extracts of the bacterial cells. The first of these studies was made by Gay and Chickering (2) who found that aqueous extracts of pneumococci, when added to homologous antiserum, yielded a precipitate which could be partially dissociated by means of dilute sodium carbonate. Such solutions contained not only type-specific agglutinins and precipitins, but protective antibodies as well. Later work by Huntoon (3) and his associates led to the belief that the immune substances were probably non-protein in nature.

Further advances in the identification of the chemical nature of antibodies have been made as a result of the extensive investigations of Felton (4). It has been shown by him that when pneumococcus antiserum is diluted with distilled water, the major portion of the antibodies separates as an insoluble precipitate. Not only can the immune bodies be precipitated by this means, but they can be effectively separated from the serum by dilution with small quantities of ethyl alcohol (5). The conditions under which antibodies can best be separated from antiserum have been thoroughly investigated (6) and the product which has been isolated has been found to have properties which definitely characterize it as a globulin more basic than ordinary serum globulin (7).

The further purification of the water-insoluble globulin bearing the immune bodies of pneumococcus antiserum has also been attempted by Felton (8). It has been shown that fractions of the protein, varying in their solubility in different



concentrations of sodium or ammonium sulfate, likewise vary in their capacity to protect mice against infection with standard doses of homologous virulent organisms. It has been shown by Felton (9), furthermore, that it is possible by the adjustment of the hydrogen ion concentration to separate from a solution of the original antibody globulin an insoluble and inert euglobulin fraction.

In summarizing the results of the more important contributions concerning the chemical nature of pneumococcus antibodies, it can now be stated with fair certainty that the latter are modified serum globulins, and that they can be separated from immune serum by a variety of methods. Although when so isolated, the antibodies are relatively pure, they are without doubt still accompanied by inert serum globulin. The present communication deals with an attempt to separate from the water-insoluble globulin of immune horse serum the biologically active protein free from accompanying inert globulins.

In his extensive studies on the proteins of normal horse serum, Soerensen (10) has found that the globulins apparently occur as labile compounds of eu- and pseudoglobulin. By means of solubility measurements Soerensen has shown that these compounds are readily dissociable, and that by repeated fractional precipitation and dialysis the constituents can be partially separated as protein fractions which become increasingly richer in eu- or pseudoglobulin, as the case may be. Although the normal horse serum globulins probably have never been obtained as pure chemical entities, yet it has been possible to dissociate these serum complexes into constituents in which one or the other of the two fractions predominates. From the results of studies on the purification and fractionation of serum globulins, both from normal and from immune horse serum, Reiner and Reiner (11) have reached similar conclusions.

The fact that the globulins of horse serum are so readily dissociable has been made use of in the present attempt to separate the immune globulin of Type I pneumococcus antiserum from accompanying inert globulins by fractionation with ammonium sulfate. The insoluble globulin fraction obtained by the dilution of antipneumococcus horse serum with distilled water, followed by the precipitation of small amounts of inert protein at pH 4.8 (8), has served as source material. By repeated fractional precipitation and dialysis this protein mixture has been separated into relatively inert euglobulin fractions, and

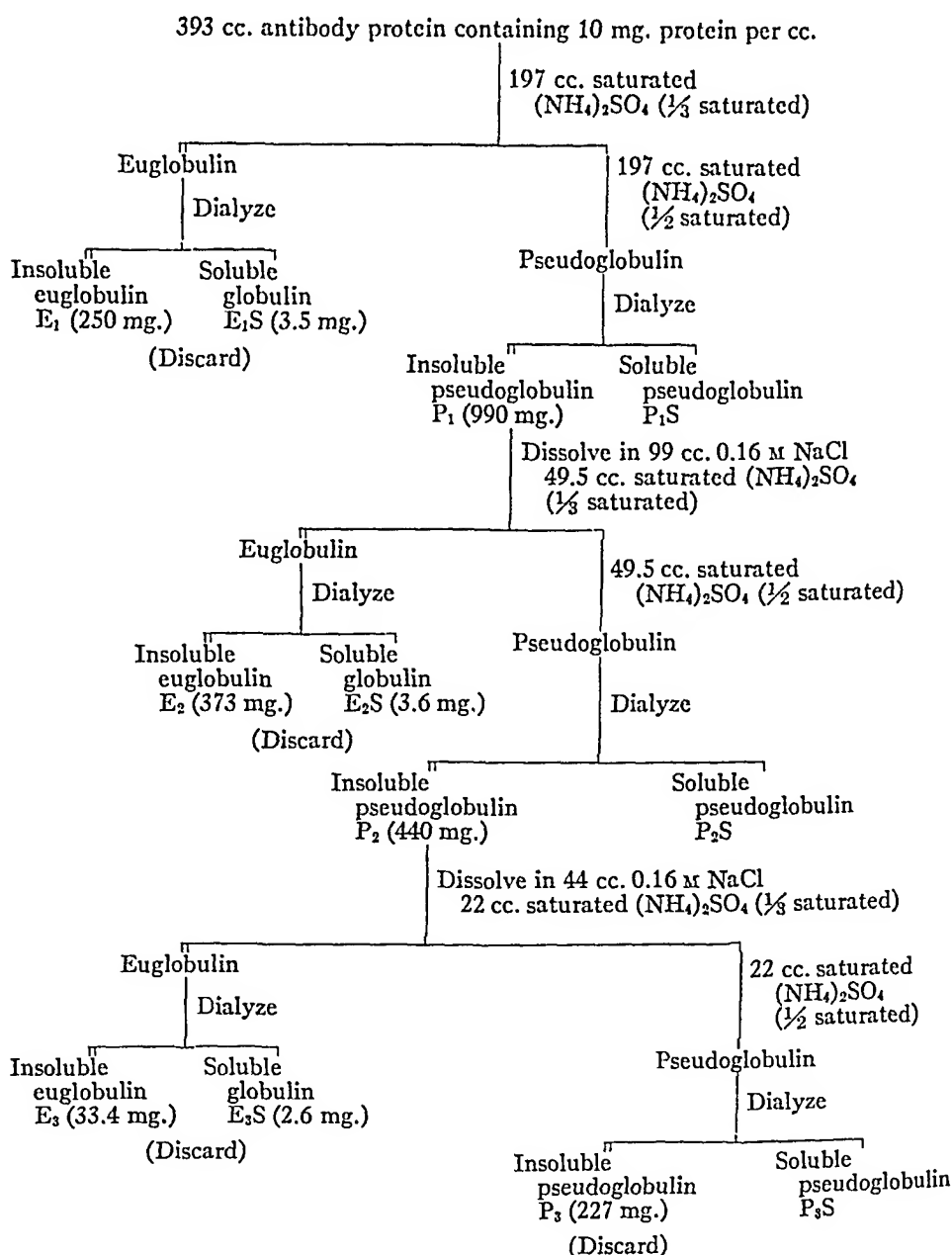
active pseudoglobulin fractions containing the major portion of the antibodies present in the starting material. A second method has also been devised for the separation of inert protein from the active immune globulin. It has been found that when pneumococcus antibody solutions, prepared according to the method of Felton (8), are treated with 0.2 M potassium acid phthalate buffer at the appropriate hydrogen ion concentration, a precipitate of inert protein is formed, leaving in solution the immunologically active globulin. The latter can be further fractionated by ammonium sulfate and dialysis to yield an end-product, which in some instances contains as much as 90 per cent of protein precipitable by the homologous type-specific polysaccharide.

When finally separated from the more inert protein, the purified immune globulin has been found considerably more potent in antibody content than the parent substance. In addition to its increased biological activity, the immune globulin possesses certain properties which distinguish it from the globulins of normal horse serum. The following experimental work is an account of the methods used in purifying the immune protein, together with a description of certain of its chemical properties. Included in this study is an investigation of certain of the chemical groupings which are believed to be involved in the union between antibody and the type-specific polysaccharide.

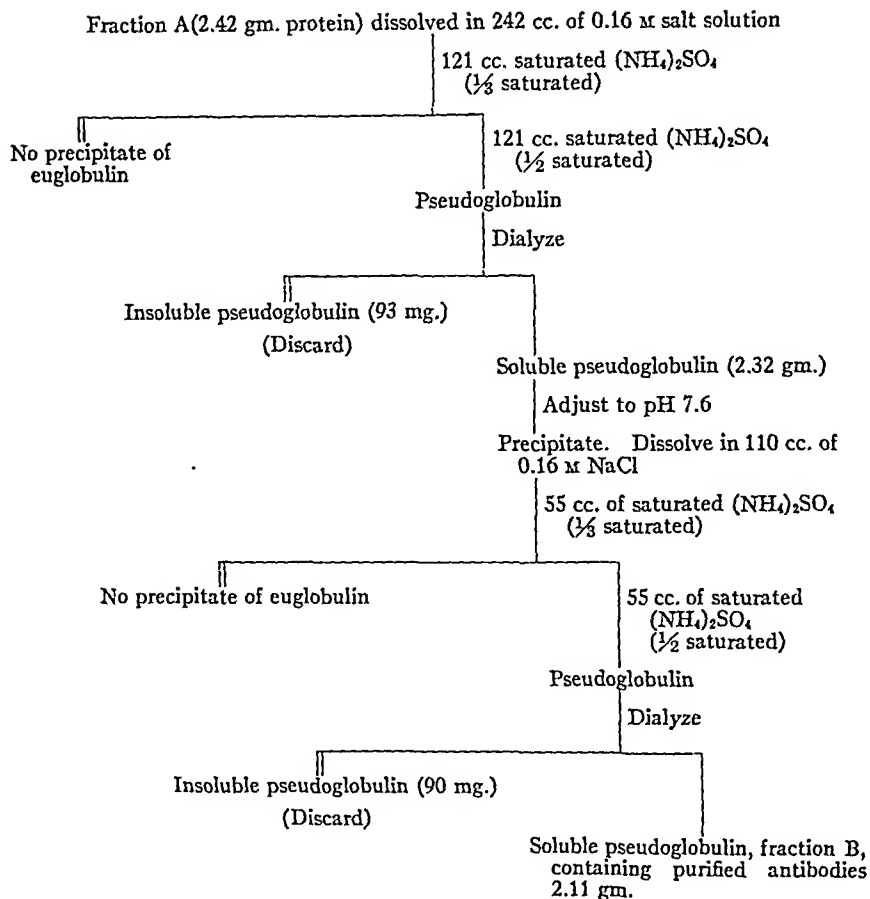
#### EXPERIMENTAL

*Preparation of Source Material.*—Type I antipneumococcus sera which have been in this laboratory since 1918 were used in the following experimental work. The method of separation of the immune globulin, which served as source material, is essentially the same as that described by Felton (8). Antibody solutions were thus obtained which varied in the amount of protein precipitable by the type-specific polysaccharide, from 25 to 42 per cent, depending upon the serum employed.

*Concentration of Type I Pneumococcus Antibody by Ammonium Sulfate.*—By repeated fractionation and dialysis it was found that the water-insoluble globulin, obtained by the dilution of antiserum with distilled water, could be separated into relatively inert euglobulin fractions, and pseudoglobulin fractions which, on dialysis, remained soluble in very dilute buffer solutions at pH 5.0. When the latter were adjusted to pH 7.6 the antibody proteins precipitated from solution, leaving small amounts of inert pseudoglobulin in the supernatant fluid. Throughout the experiment, the various protein fractions were dialyzed against 0.005 M acetate buffer at pH 5.0. The method of fractionation is summarized in the following outline.



Fractions  $\text{P}_1\text{S}$ ,  $\text{P}_2\text{S}$ , and  $\text{P}_3\text{S}$ , containing the major portion of the antibodies, were now combined and the hydrogen ion concentration of the solution adjusted to 7.6 with 0.1 M NaOH. The antibody proteins (designated as fraction A) were thus precipitated, leaving in solution 0.077 gm. of water-soluble inert globulin. Fraction A, the antibody protein, was now dissolved in 242 cc. of 0.9 per cent salt solution and further fractionated as follows:



By this method of fractionation it is possible to separate the source material into relatively inert euglobulin fractions, and a pseudoglobulin fraction with marked biological activity as determined by quantitative precipitin titration (12). This immune protein, in addition to its immunological reactivity, has certain other properties which distinguish it sharply from the source material and from the globulins of normal horse serum.

When the above method of fractionation was followed, the actual quantities of the inert protein which separated from the active pseudoglobulin showed wide variation with different lots of immune serum. The immunological activity, however, of the pseudoglobulin isolated

from different lots of antisera was invariably the same provided the conditions of fractionation were carried out as described. Thus from an antiserum, which on dilution yielded a water-insoluble globulin containing only 25 per cent precipitable protein as determined by the quantitative precipitin titration, a pseudoglobulin fraction containing 65 per cent precipitable protein was finally obtained. On the other hand, the immune protein obtained from a more potent antiserum, having initially 42 per cent precipitable protein, yielded a purified antibody containing 67 per cent precipitable protein.

*Concentration of Type I Pneumococcus Antibody by Precipitation of Inert Globulin with Potassium Acid Phthalate Buffer.*—By means of the foregoing procedure it has been possible to enhance the activity of the antibody so that approximately two-thirds of the protein is precipitable by the type-specific polysaccharide. This method of concentration, however, yields an end-product in which serologically inert proteins still persist, and which cannot be separated by refractionation with ammonium sulfate.

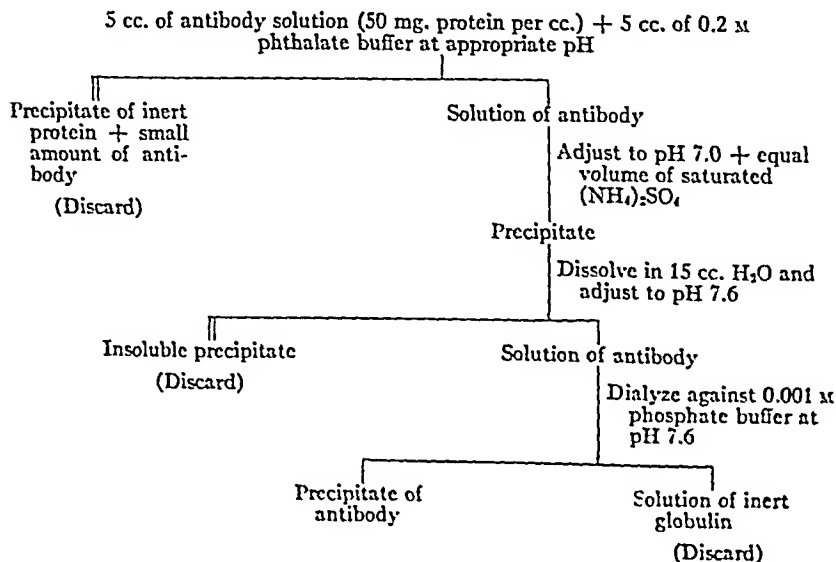
It has been found, however, that when a concentrated solution of the source material is treated with an equal volume of 0.2 M potassium acid phthalate buffer at a definite acid pH, which must be precisely determined for any given lot of antibody solution, an inert protein fraction separates as a precipitate, leaving in solution the major portion of the active antibodies. When the latter are precipitated by half saturation with ammonium sulfate, and the precipitated protein dialyzed at pH 7.6 against 0.001 M phosphate buffer, the antibody protein separates in a high state of purity. Thus it is possible to secure a solution of immune globulin containing between 75 and 90 per cent specifically precipitable protein, depending upon the serum which served as the original source material.

In order to establish the optimum conditions for the precipitation of the inert protein fraction, it is necessary, with each new lot of source material, to determine the appropriate hydrogen ion concentration of the buffer to be employed according to the following method.

100 cc. of 0.2 M potassium acid phthalate solution were measured into each of four flasks. To each were added 5.30, 8.66, 12.00, and 15.95 cc. of 0.2 M HCl solution respectively. The pH of each solution was found to be approximately

3.8, 3.7, 3.6, and 3.4 respectively. Into each of four 50 cc. centrifuge tubes were now measured accurately 5 cc. of antibody solution (prepared by the method of Felton (8)) containing 50 mg. protein per cc. To each tube was then added, with stirring, 5 cc. of the phthalate buffer solutions at the different hydrogen ion concentrations. The tubes were allowed to stand at room temperature for 5 minutes, and the precipitates centrifuged off and discarded. The clear supernatant liquids were neutralized cautiously with 1.0  $M$  NaOH. To each solution was added an equal volume of saturated ammonium sulfate solution, and the precipitates collected by centrifugation. The precipitates were dissolved as completely as possible in 15 cc. of water, and in each tube the pH adjusted to 7.6. (It was observed, at this point, that in several of the tubes some of the protein failed to dissolve. It was later found that this phenomenon occurred only in those tubes where the conditions for the maximum precipitation of inert protein were not optimal.) The solutions were clarified by centrifugation and to each supernatant liquid was added 1 cc. of 0.1  $M$  phosphate buffer at pH 7.6. The solutions were dialyzed at 5°C. against 0.001  $M$  phosphate buffer at pH 7.6 until free from sulfate ions. The active insoluble antibody protein which separated on dialysis, was recovered by centrifugation. The supernatant liquids, containing small quantities of inert protein, were discarded. The precipitates of immune protein were dissolved in 15 cc. of saline and the activity of the antibody was ascertained by means of the quantitative precipitin titration.

The method of fractionation can be diagrammatically represented as follows:



Quantitative protein determinations of the various antibody fractions precipitated at different hydrogen ion concentrations, and the immunological activity of the purified antibody protein are given in Table I.

From the results given in Table I, it can be seen that when the pH of the phthalate buffer is varied in increments as small as 0.1 of a pH unit the activity and yield of the purified antibody shows wide variation. This can be accounted for only by the fact that when the pH of the buffer is not suitable, the separation of active and inert protein is incomplete. It is of utmost importance, therefore, to establish the optimum condition for the precipitation of inert protein for each antibody solution under investigation before attempting to purify the entire solution. When these conditions have been established, the

TABLE I

*Effect of Change in pH of 0.2 M Potassium Acid Phthalate Buffer on the Purification of Type I Pneumococcus Antibody*

| pH  | Amount of antibody protein recovered | Amount of protein precipitable by acetyl polysaccharide in recovered antibody protein fraction |
|-----|--------------------------------------|--|
|     | <i>per cent</i>                      | <i>per cent</i>  |
| 3.4 | 13                                   | 48   |
| 3.6 | 25                                   | 61   |
| 3.7 | 37                                   | 76   |
| 3.8 | 27                                   | 64   |

remaining antibody solution can be rapidly purified. Thus one obtains ultimately a solution of purified antibody containing approximately 75–80 per cent of the total antibody present in the starting material, and containing between 75 and 90 per cent type-specific precipitable protein, depending upon the serum employed. From the water-insoluble antibody obtained from three separate lots of anti-serum, containing originally 35, 30, and 43 per cent specifically precipitable protein, we obtained purified antibody solutions having 76, 85, and 90 per cent specifically precipitable protein respectively.

When antibody solutions are purified by the above method, it has been found that several factors other than hydrogen ion concentration influence the precipitin titre of the end-product. Thus it has been observed that if the initial concentration of water-insoluble globulin

is varied, or if the precipitation with buffer is carried out at 0° instead of at room temperature, the percentage of protein precipitable by the type-specific polysaccharide is considerably decreased. In conclusion it may be said that the separation of inert globulin cannot be accomplished merely by adjusting the hydrogen ion concentration of the antibody solution to the appropriate value with acid, nor can it be accomplished by substituting acetate buffer for the phthalate buffer. The phthalate buffer appears to have a selective precipitating action upon the inert globulin. Felton (13) has also pointed out that inert globulin can be selectively precipitated from antibody solutions by certain metallic salts such as aluminum and zinc chlorides to yield an end-product of which 80 to 90 per cent of the total protein is precipitable by the type-specific carbohydrate.

*Biological Activity of the Various Protein Fractions Obtained by the Concentration of Pneumococcus Antibody*

The usual method of ascertaining the potency of pneumococcus antibody solutions is to determine the minimum amount necessary to protect mice against a standard dose of virulent pneumococci of the homologous type. In attempting to quantitate small increments of increase in the biological activity of antibody solutions this particular method is, for obvious reasons, inadequate. However, the quantitative measurement of type-specific precipitins as devised by Heidelberger and Kendall (12), affords an admirable means of determining small differences in the biological activity of pneumococcus antibody solutions. The adoption of this method has made it possible to follow the purification of the immune proteins, and to determine the activity of the protein fractions discarded during the fractionation process.

Thus the percentage of protein precipitable by the type-specific carbohydrate (acetyl polysaccharide of *Pneumococcus* Type I (14)) was determined in the discarded fractions E<sub>1</sub> and E<sub>2</sub> (Method I) and compared with the activity of the purified immune protein. The method of determining the maximum precipitable protein was as follows:

The fraction under investigation was so diluted that each cc. contained exactly 2.0 mg. of protein. Four samples of 5 cc. were pipetted into each of four centrifuge tubes. The latter were cooled to 0°C. and to each were added from a cali-



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The fraction under investigation was so diluted that each cc. contained exactly 2.0 mg. of protein. Four samples of 5 cc. were pipetted into each of four centrifuge tubes. The latter were cooled to 0°C. and to each were added from a cali-

brated pipette, 5 cc. of a solution of the type-specific polysaccharide at 0°C. The concentration of polysaccharide was so chosen that the first tube contained a total of 0.5 mg., the second 0.2 mg., the third 0.1 mg., and the fourth tube 0.05 mg., of type-specific carbohydrate. The tubes were kept at 0°C. for 48 hours. The immune precipitate was removed by centrifugation at 0°C. and washed twice with 3 cc. portions of ice cold saline. The clear supernatant liquids were discarded, and the precipitated immune protein dissolved in a few cc. of 0.1 M NaOH. The solutions were next transferred quantitatively to Kjeldahl flasks, and the analysis for protein nitrogen was carried out in the usual way (15).

Thus for each fraction of protein investigated, the total content and percentage of active protein precipitated by the type-specific carbohydrate were determined. The activity of the various protein fractions isolated during the purification of antibody by the ammonium sulfate method is shown in Chart 1.

From the results given in the chart it can be seen that 42 per cent of the protein in the original antibody solution was precipitable by the type-specific carbohydrate. When subjected to repeated fractionation by means of ammonium sulfate, the amount of precipitable nitrogen was increased to a maximum of 67 per cent. By the method of fractionation with ammonium sulfate, it has not been found possible to increase the amount of precipitable protein beyond the value of 67 per cent. Although it has not been indicated on Chart 1, it has been found that a solution of water-insoluble globulin obtained from an antiserum of low titre and containing originally not more than 25 per cent specifically precipitable protein, can be concentrated by the method so that the final activity of the immune protein thus derived equals that of the protein obtained from the concentration of more potent source material.

Although the quantity of active protein discarded in fractions  $E_1$  and  $E_2$  is in each instance approximately half that of the source material, the actual quantity of immune protein lost represents only a small percentage of the total. It is of interest to note that the ammonium sulfate method of fractionation outlined above has never yielded an end-product containing more than 67 per cent of type-specific precipitable protein. The reason for this probably lies in the fact that there remains in the concentrated antibody an inert globulin intimately associated with the active constituent and inseparable from it by the

method described. It can be seen from Chart 1 that when this antibody is further purified by precipitation with potassium acid phthalate buffer, a considerable quantity of inert globulin is eliminated, and the activity of the antibody is increased to a value of 88 per cent type-specific precipitable protein. The remaining 12 per cent of protein

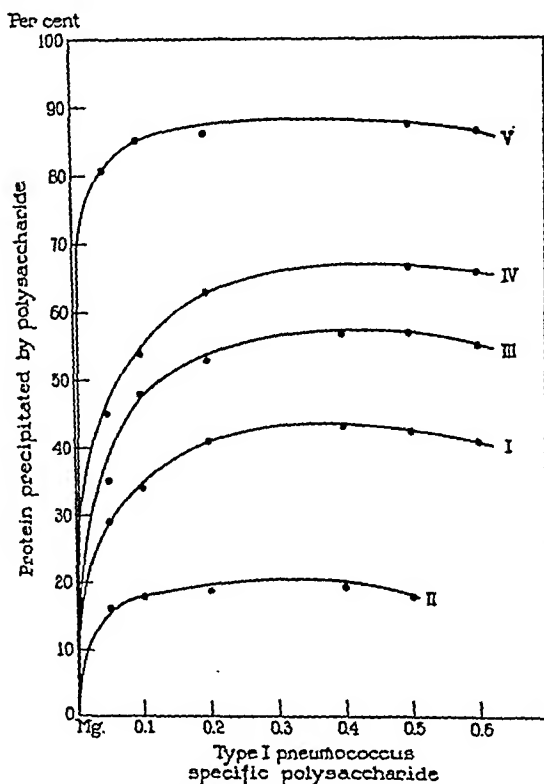


CHART 1. I = original antibody solution. II = fraction E<sub>1</sub> and E<sub>2</sub> (ammonium sulfate method). III = fraction A (ammonium sulfate method). IV = fraction B (ammonium sulfate method). V = fraction B after being subjected to purification by potassium acid phthalate buffer method.

may possibly be represented by other antibodies present in the original serum. It must be remembered that in antipneumococcus serum there are a number of antibodies reactive with constituents of the cell other than the capsular polysaccharide, and that the relative quantities of these antibodies vary from serum to serum. The protein precipitable

by the type-specific polysaccharide does not therefore represent the total antibody protein present in the concentrated mixture. It can be clearly demonstrated experimentally that the latter still contains antibodies for the cellular proteins of the pneumococcus and for the somatic carbohydrate as well (16).

*Properties of the Immune Protein Recovered from Antipneumococcus Serum Type I*

Both Felton and Reiner and Reiner have observed that the isoelectric point of the water-insoluble globulin isolated by them from pneumococcus antiserum is distinctly more alkaline than is that of normal horse serum globulin. This property becomes even more striking and more sharply defined when the inert protein present in the globulin studied by these investigators is separated by the methods outlined above. The unusual alkaline isoelectric point of the purified immune proteins is perhaps its most characteristic physical property, one which may possibly be explained by the relatively high ratio of amino to carboxyl groups present in the protein molecule. In the absence of high concentrations of electrolytes the immune protein is insoluble in distilled water at its isoelectric point. The protein may be brought into solution by the addition of very dilute alkali or acid, or by bubbling carbon dioxide into an aqueous suspension of the protein. The antibody can be quantitatively precipitated from such solutions by adjusting the hydrogen ion concentration again to 7.6. This point of minimum solubility may be tentatively regarded as the isoelectric point of the antibody protein, although the migration of particles in a cataphoretic field has as yet not been observed. Unlike the globulins of normal horse serum, the immune globulin showed only 14.85 per cent of nitrogen when analyses were performed on dried, ash-free, samples of the material. Quantitative analyses have shown that the antibody protein contains approximately 2.75 per cent of lipoid (17) and a prosthetic carbohydrate grouping as revealed by the qualitative Molisch test; the immune globulin, therefore, cannot be regarded as a simple protein.

*Distribution of Basic Amino Acids in the Immune Protein of Antipneumococcus Serum Type I*

In view of the fact that the immune protein derived in the manner described has an isoelectric point lying so far in the alkaline range, it might be anticipated that the protein would contain a higher percentage of basic amino acids than would the globulin of normal serum. In order, therefore, to ascertain whether the purified antipneumococcus

TABLE II

*Distribution of Basic Amino Acids in the Globulins of Normal and Immune Horse Serum*

| Amino acid determined | Normal serum globulin (total)<br>Cavett's method | Concentrated pneumococcus antibody |                 | Average         |
|-----------------------|--|------------------------------------|-----------------|-----------------|
|                       |  | Analysis 1                         | Analysis 2      |                 |
|                       | <i>per cent</i>                                  | <i>per cent</i>                    | <i>per cent</i> | <i>per cent</i> |
| Amide N.....          | 9.46   | 9.22                               | 9.21            | 9.22            |
| Humin N.....          | 2.26   | 2.28                               | 2.08            | 2.18            |
| Cystine N             |  |                                    |                 |                 |
| (a) precipitate.....  | 0.31   | 0.53                               | 0.38            | 0.46            |
| (b) filtrate.....     | 0.78   | 0.71                               | 0.88            | 0.80            |
| (c) total.....        | 1.09   | 1.24                               | 1.26            | 1.25            |
| Arginine N.....       | 8.98   | 9.02                               | 8.58            | 8.80            |
| Histidine N.....      | 5.85   | 5.55                               | 6.40            | 5.98            |
| Lysine N.....         | 8.31   | 9.90                               | 10.04           | 9.97            |
| Filtrate N            |  |                                    |                 |                 |
| (a) amino.....        | 59.18  | 59.60                              | 59.16           | 59.38           |
| (b) non-amino N.....  | 6.37   | 3.90                               | 4.67            | 4.29            |
| Total N.....          | 100.7  | 100.0                              | 100.5           | 100.3           |

protein shows any marked difference in the distribution of basic amino acids, an analysis was performed according to the Van Slyke method as modified by Cavett (18). The results of these analyses are given in Table II.

From the results given in Table II it can be seen that aside from a slightly higher lysine content there appears to be no essential difference in the distribution of the basic amino acids of the concentrated pneumococcus antibody globulin as compared with normal serum.

*Nature of the Specific Groups Involved in the Union between Type-Specific Antibody and the Capsular Polysaccharide of Type I Pneumococcus*

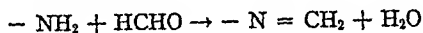
In view of the marked alkaline isoelectric point of the immune protein, it seemed not unlikely that the free amino groups of the antibody protein might play some rôle in the union of the antibody molecule with the specific polysaccharide of Type I Pneumococcus. To investigate this possibility, one of the hydrogen atoms of the primary amino groups of the immune protein was replaced by an acetyl radical under conditions in which the protein was not denatured.

Thus 0.5 gm. of immune globulin was dissolved in 100 cc. of 0.16 M NaCl solution and 30 gm. of sodium acetate were added. Ketene gas, generated by the pyrolysis of acetone (19), was bubbled through the solution at 0°C. for 60 minutes. During this period the pH of the solution did not go lower than 5.0. After the acetylation was completed, the protein was precipitated from solution by saturation with solid sodium acetate. The precipitated protein was separated by centrifugation, and dialyzed until free from electrolytes. The solution was then analyzed for total nitrogen content. Amino nitrogen determinations revealed the fact that no free amino groups could be detected. An acetyl determination (20) performed on a dried sample of the ash-free acetylated protein showed that the latter contained one acetyl group for every primary amino group present in the original protein molecule. The acetylated protein in varying concentration was tested for the presence of type-specific precipitins reactive with the acetyl polysaccharide.

From the results of the qualitative precipitin titrations given in Table III it is seen that, by replacing one hydrogen atom of the primary amino groups with an acetyl radical, the immune protein loses to a great extent its capacity to precipitate the type-specific polysaccharide. It can also be seen that when relatively high concentrations of specific polysaccharide are added to the acetylated antibody a precipitate is formed. Although the amino groups of the immune protein may be involved in the union of antibody and the carbohydrate, yet it is evident from Table III that certain other reactive groups must likewise play a part. It is believed, however, that the amino groups in the native antibody protein molecule are concerned in the union of antibody with specific carbohydrate. Support for this view is provided by the results of the following experiments in which

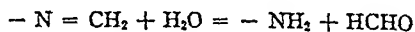
the serological reactivity of the antibody is completely lost when a solution of the immune protein is treated with formaldehyde (21).

The reaction between the free amino groups of protein and formaldehyde presumably takes place according to the following equation.



Thus 150 mg. of antibody protein, dissolved in 1.5 cc. of 0.85 per cent NaCl solution were treated with 1.5 cc. of normal  $NaHCO_3$  solution. The mixture was cooled to  $0^\circ C$ . 0.75 cc. of 37 per cent neutral formalin was added and the solution was allowed to stand for 30 minutes at  $0^\circ C$ . The solution was diluted with 15 cc. of ice water, and rapidly dialyzed against cold water, until the dialysate no longer showed the presence of free formaldehyde when tested with silver nitrate and ammonia solution. After the addition of the proper concentration of sodium chloride the clear solution of formalized antibody protein gave no precipitin test in the presence of the type-specific polysaccharide. The serological specificity of the antibody protein could be restored, however, by adjusting the pH of the formalized protein to 4.0 and allowing the mixture to stand at  $0^\circ C$ . for several days. In Table IV may be seen the comparative results of the precipitin titrations of original antibody, formalized antibody, and the "deformalized" antibody.

From the results of the qualitative precipitin reactions given in Table IV, it can be seen that when the antibody protein is treated with formaldehyde the serological specificity is lost. This loss is presumably due to the fact that the free amino groups of the immune protein molecule are converted to the grouping  $C=NH_2$ . It has been well established that this grouping is stable only when the hydrogen ion concentration of the solution of formalized protein is maintained above 7.0. When the pH of the solutions is lowered the derivative presumably reacts with water and restores the grouping originally present according to the reaction



The reaction of concentrated formaldehyde upon native protein, however, is without doubt not as simple as presented above. It is not improbable that other changes take place within the sensitive protein molecule when in contact with high concentrations of the reagent. Although the nature of such changes is not understood, it is significant that when the formalized protein is subjected to the influence of hydrogen ions, the amino groupings are restored, and the serological



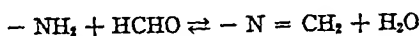
TABLE III  
*Precipitin Reactions of Acetylated and Native Pneumococcus Antibody*

| Solution of pneumococcus antibody | Concentration of protein per cc.<br>mg. | Final dilution of Type I pneumococcus acetyl polysaccharide |         |         |          |          |          |           |           |           |             |
|-----------------------------------|---|---|---------|---------|----------|----------|----------|-----------|-----------|-----------|-------------|
|                                   |   | 1:2,000   | 1:4,000 | 1:8,000 | 1:16,000 | 1:32,000 | 1:64,000 | 1:128,000 | 1:256,000 | 1:512,000 | 1:1,024,000 |
| Native                            | 5.0                                     | ++  | ++++    | ++++    | ++++     | ++++     | ++++     | ++++      | ++++      | ++++      | ++++        |
| Acetylated                        | 5.0                                     | +±  | +±      | +       | ±        | 0        | 0        | 0         | 0         | 0         | 0           |
|                                   | 1.0                                     | ++++  | ++++    | +       | 0        | 0        | 0        | 0         | 0         | 0         | 0           |
|                                   | 0.1                                     | ++  | +±      | +       | 0        | 0        | 0        | 0         | 0         | 0         | 0           |

TABLE IV  
*Precipitin Reactions of Native, Formalized, and Deformalized Type I Pneumococcus Antibody*

| Substance tested           | Concentration of protein per cc.<br>mg. | Final dilution of Type I pneumococcus acetyl polysaccharide |         |         |          |          |          |             |
|----------------------------|---|---|---------|---------|----------|----------|----------|-------------|
|                            |   | 1:2,000   | 1:4,000 | 1:8,000 | 1:16,000 | 1:32,000 | 1:64,000 | 1:1,024,000 |
| Original antibody.....     | 5                                       | ++  | ++++    | ++++    | ++++     | ++++     | ++++     | ++++        |
| Formalized antibody.....   | 5                                       | 0   | 0       | 0       | 0        | 0        | 0        | 0           |
| Deformalized antibody..... | 5                                       | +±  | +±      | ++++    | ++++     | +±       | +±       | +           |

specificity of the immune protein is partially regained. It is likewise significant that the "deformalized" protein does not regain its full specificity under these conditions. Whether this is due to the fact that the reaction



is not completely reversible, or whether other chemical changes have taken place within the immune protein molecule during its contact with concentrated formaldehyde cannot be answered with certainty.

*Immunological Changes Accompanying the Esterification of the Deacetylated Polysaccharide of Type I Pneumococcus*

Studies carried out in this laboratory on the chemical nature of the type-specific polysaccharide derived from encapsulated microorganisms have revealed that all the specific carbohydrates thus far investigated contain uronic acids as constituents of the polysaccharide molecule. The occurrence of glucuronic acid or its isomers in these bacterial products suggests the possibility that the highly polar carboxyl group of the uronic acid and its stereochemical relationship to other groups in the polysaccharide molecule might not only determine to a considerable extent the specificity of the carbohydrate, but that the carboxyl itself might actually enter into chemical combination with homologous antibody when the two substances are brought together either *in vivo* or *in vitro*. Consequently, if the carboxyl group of the Type I specific carbohydrate were to be covered with an ester group the derivative either should no longer react with homologous antibody, or the reaction should be greatly diminished. In order to test the validity of this hypothesis, the following experiments were performed.

0.95 gm. of dried deacetylated Type I polysaccharide was suspended in 10 cc. of anhydrous ether and treated with 30 cc. of an ethereal solution of diazo methane, prepared from 3 cc. of nitrosomethyl urethane. The mixture was shaken at room temperature for 24 hours, and the precipitate was then filtered and washed with anhydrous ether. 1.00 gm. of substance was recovered. Analysis of the end-product of the reaction showed that the material contained 11.2 per cent methoxyl and only 0.3 per cent primary amino nitrogen. The substance was found to be soluble in water, and such solutions were quite neutral, indicating the absence of

free carboxyl groups. This material, when tested with Type I antipneumococcus serum, failed to precipitate, as indicated in Table V.

From the results of the precipitin tests it is seen that when the carboxyl group of the specific polysaccharide is esterified by means of diazo methane, the resulting methyl ester of the polysaccharide fails to precipitate in homologous antipneumococcus serum within the range of dilutions tested. It appears, therefore, that the presence of the carboxyl group in the type-specific polysaccharide is essential for complete specific union and precipitation with homologous antibody.

TABLE V

*Precipitin Reactions of Original, Esterified, and Saponified Forms of Type I Pneumococcus Polysaccharide in Homologous Antipneumococcus Serum*

| Substance tested          | Final dilution of substance tested |         |         |          |          |          |           |
|---------------------------|------------------------------------|---------|---------|----------|----------|----------|-----------|
|                           | 1:2,000                            | 1:4,000 | 1:8,000 | 1:16,000 | 1:32,000 | 1:64,000 | 1:128,000 |
| Original* polysaccharide  | +++±                               | ++++    | ++++    | ++++     | ++++     | ++++     | +++       |
| Esterified polysaccharide | ±                                  | ±       | 0       | 0        | 0        | 0        | 0         |
| Saponified polysaccharide | -†                                 | -†      | ++++    | ++++     | ++++     | ++++     | +++±      |

\* Deacetylated form of Type I specific polysaccharide.

† Not done.

When the esterified polysaccharide is treated with dilute alkali at 100°C., the methyl ester grouping is removed as may be seen from the following experiment.

488.5 mg. of ester were dissolved in 20 cc. of water and 1 drop of 1 per cent phenolphthalein solution was added. 10 cc. of 0.1 N NaOH were added and the mixture was heated on a water bath for 30 minutes. The solution was cooled, and the excess NaOH was neutralized with 1.9 cc. of 0.1 N HCl. The neutral solution of polysaccharide was dialyzed until free from chloride ion, and then poured into 10 volumes of acetone. The precipitated polysaccharide was removed by centrifugation and dried. This substance was acidic in character, and had a specific optical rotation of +277.5°. The material had an acid equivalent of 580 when titrated to pH 7.0. The substance contained 4.82 per cent of nitrogen, and only 0.39 per cent of primary amino nitrogen. A methoxyl determination<sup>1</sup>

<sup>1</sup> We wish to thank Mr. Frank H. Babers for assisting with the micro analytical determinations.

revealed the fact that the saponified derivative now contained only 5.21 per cent of methoxyl. From the results of the analysis of the esterified and the saponified products, it can be concluded that the former substance contains a methyl grouping bound on the primary amino group of the parent substance, a methoxyl group attached probably to one of the hydroxyl groups, as well as a methyl ester group coupled to the carboxyl grouping of the uronic acid. When the methylated compound is warmed with dilute alkali, the ester grouping is removed, and the resulting derivative still contains a methyl group substituted on the primary amino group, as well as a methoxyl group still covering an hydroxyl group. The latter compound, however, unlike the serologically inert ester, reacts readily with antiserum as can be seen in Table V.

It is obvious, therefore, that the presence of a free carboxyl group is of primary importance in rendering the Type I polysaccharide serologically reactive, and that the substitution of the  $\text{NH}_2$  and  $\text{OH}$  groups in the polysaccharide molecule has a less pronounced influence upon serological specificity.

#### DISCUSSION

The earlier hypotheses concerning the nature of immune bodies have gradually given way to evidence accumulated largely during the past decade which at present definitely points toward the fact that the circulating antibodies are in reality modified serum globulins. Because the gross qualitative chemical tests for the detection of protein in biologically active fluids are so greatly exceeded in delicacy by the more subtle immunological tests, it is our opinion that the chemical evidence fails to support the hypothesis concerning the non-protein nature of antibodies. For years an analogous situation has existed in the attempt to define the chemical nature of enzymes, yet it has been only recently that the nature of these substances has been defined with certainty. An approach quite different from the absorption methods hitherto employed in the concentration of enzymes has made possible the isolation of crystalline proteins possessing the biological activity of crude enzyme mixtures. These crystalline proteins show both constant physical properties and biological action on repeated crystallization (22). The success of Northrop and his associates in isolating from crude enzyme solutions a crystalline protein possessed with unique chemical and biological properties has served as a stimulus to us in attempting to isolate the immune protein of antipneumococcus

serum free from inert constituents. That this objective has at least in part been achieved can be seen from the results of the foregoing experiments.

The precise method of Heidelberger and Kendall which permits the determination of small differences in activity of protein fractions has been used in this study as a means for determining the potency of antibody solutions. Although the quantity of immune precipitate formed by a given weight of type-specific polysaccharide is the measure of only one of the active constituents of antibody solutions, yet this measurement serves as an index of the activity of antibody concentration as a whole. The solubility properties of the other antibody proteins parallel so closely those of the type-specific immune body, that the former are found intimately associated with the latter and relatively more concentrated, as the purification progresses.

Many samples of water-insoluble globulin obtained by the dilution of antipneumococcus serum have been subjected to the methods of fractionation outlined in the experimental part of this communication. Although the initial concentration of the type-specific antibody in different samples of source material has been found to vary from 25 to 42 per cent, yet invariably the end-product never attains a value of 100 per cent of type-specific precipitable protein. The reason for this may lie in the fact that the remaining proteins not precipitable by the type-specific polysaccharide represent the other antibodies, and possibly small amounts of inert globulin which cannot be separated by the methods of fractionation employed.

One of the most striking observations encountered during the course of the experimental work was the fact that the purified antibody protein was found to have essentially the same percentage of basic amino acids as has the globulin of normal horse serum. Although the proportion of these amino acids is in each instance approximately the same, the normal globulin is biologically inert, whereas the immune protein possesses both chemical and immunological properties which distinguish it sharply from the former. By simple chemical experiments we have attempted to show that the biological activity of pneumococcus antibody protein is to a great extent dependent upon the presence of free amino groups in the protein molecule. When these groups are covered with a chemical radical the activity of the protein

molecule is lost. It has been further shown that upon removal of the substituted group the antibody molecule again becomes specifically reactive. Such experiments, however, fail to account for the immunological specificity which the protein exhibits. A possible explanation of the origin and specificity of antibodies has been suggested independently by Mudd (23) in this country, and by Breinl and Haurowitz (24) in Germany. In the opinion of these investigators, the normal course of serum globulin synthesis is altered in the animal body when antigen reaches the site at which synthesis takes place. Under the influence of the antigen the serum globulin is altered in a way characteristic for the foreign stimulus. When the modified globulin eventually encounters the antigen either in the circulation or *in vitro* interaction of the two is possible.

From the results of studies on the experimental transformation of specific types of *Pneumococcus* (25) it seems not unlikely that the synthesizing function of a cell can be specifically oriented by a given stimulus. When R forms of pneumococci, irrespective of the type from which they were derived, are grown in the presence of a specific activator contained in a cell-free extract of the S organism, the former are induced to synthesize the capsular polysaccharide of the same type as that from which the bacterial extract was prepared. If the nature of the activator is changed, however, the same R organism can be made to synthesize a capsular polysaccharide quite different in chemical constitution and in biological specificity from that which it produced when stimulated by the original activator. Thus it is seen that bacterial cells under the same general environmental conditions can, by means of specific stimuli, be directed to synthesize polysaccharides which are chemically distinct and biologically specific.

Although it is probable that the union of antibody with type-specific polysaccharide involves the interaction of polar groups of opposite charge, yet this concept alone does not explain the specificity of the reaction. There is, however, certain evidence to support the view that the specificity of serological reactions is governed by the arrangement in space of the polar groups of the reactive substances. It has been shown in this laboratory (26) that antigens prepared from the azo phenol glycosides of glucose and galactose react only in their homologous antisera. In respect to the number and nature of polar

groups these two glycosides are identical. Since the active groups are in each instance the same, the mechanism underlying the union of each glycoside with its homologous antibody must likewise be the same. The specificity of this reaction therefore can be accounted for only by known differences in the spatial arrangement of the polar groups of the fourth carbon atom of each glycoside. In their studies on the serological differentiation of steric isomers Landsteiner and van der Scheer have arrived at similar conclusions (27). Since the spatial arrangement of identical polar groups suffices to determine serological specificity, it therefore seems justifiable to assume in the case of the type-specific antibody of *Pneumococcus* that the spatial arrangement of the polar groups in the immune protein may likewise determine its specific capacity to react with the polysaccharide of the homologous type.

In view of these considerations it is believed that the general mechanism underlying the union of antibody and carbohydrate involves the interaction of polar groups of opposite charge. Similar views have indeed been expressed by Heidelberger and Kendall (28), by Marrack (29), and by Haurowitz and Breinl (30). In the case of the *Pneumococcus* Type I carbohydrate it is believed, on the basis of the evidence presented, that the carboxyl groups of the polysaccharide are the dominant groups which interact to form the immune precipitate. Whether the carboxyl groups actually combine with the amino groups of the antibody protein and whether the formation of an insoluble precipitate involves further chemical change in the protein molecule, such as a specific denaturization, cannot be answered with certainty at the present time. It is believed, however, that the specificity of this reaction is determined by the stereochemical relationship of the dominant polar groups in the reacting molecules, whether they be antigen or antibody. If the spatial pattern of the polar groups of both antigen and antibody is of exactly the correct order, then union occurs. If, however, this relationship is disturbed by artificial means, as has been experimentally demonstrated by covering the dominant polar group of either polysaccharide or antibody with a chemical radical, the pattern is destroyed and union between them is either greatly modified or fails to take place. When the original constitution of the reacting substances is restored, however, serological specificity is regained.

In conclusion the authors wish to express their grateful appreciation of the helpful suggestions and criticisms of Dr. John H. Northrop and Dr. Oswald T. Avery.

#### SUMMARY

1. Methods for the concentration of Type I pneumococcus antibody have been outlined.
2. The physical and chemical properties of the purified antibody have been described.
3. The chemical basis of serological specificity has been discussed.

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# PATHOGENIC ORGANISMS OF THE GENUS *LISTERELLA*

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PLATES 11 AND 12

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During the past ten years, a number of observers have isolated small Gram-positive bacilli from a variety of diseases in widely different host species including man. With the exceptions noted below these organisms have not been classified or correlated. In the course of studies on a disease in fowl characterized by massive necrosis of the myocardium with which a similar organism is associated, relationships were observed which it is the purpose of this study to present.

Murray (1), in 1926, isolated an organism from a disease in his stock rabbits in Cambridge. He was unable to classify it with any existing genus and since one of the predominant features of the disease was a marked mononucleosis, he called the organism *Bacterium monocytogenes*. Pirie (2), from a plague-like disease in the gerbille in South Africa isolated an organism similar to Murray's. He proposed the name *Listerella hepatolytica*, but in view of the blood response to the organism, which was not observed until after Murray's description appeared, he considers "*monocytogenes*" more suitable as a specific name. In Bergey's Manual these two species are listed under the genus "*Listerella*."

In 1931 Gill (3), in New Zealand, reported the isolation of small Gram-positive bacilli from the brains of sheep suffering from an epizootic mid-brain encephalitis known as "circling." He suggests that the disease is widespread, similar cases having occurred in West Australia, and in Indiana in the United States. Ten Broeck obtained organisms from sporadic cases of encephalitis in sheep occurring in the New Jersey vicinity which were culturally and morphologically similar to those described by Gill and did not fit any known genus. TenBroeck's observations were not reported.

Beginning in 1930, Jones and Little studied cases of bovine encephalitis occurring sporadically in New Jersey. From the brains of cattle dead of the disease a similar organism was isolated. A note on this work was published in 1934 (4). They compared strains of their organism with the sheep organism isolated by TenBroeck, and found them to be the same. This latter observation, however,

was not reported. Schultz (5), in 1934, reported the repeated isolation of a similar organism from a case of meningo-encephalitis in a woman in California. In 1934, Burn (6), in New Haven, obtained an unclassifiable bacillus from two cases of purulent meningitis in new-born infants and, later, from a case in an adult. He suggested a relationship with *B. monocytogenes* on the basis of morphological similarity. An unpublished comparison made by Burn showed his human strain to be identical with a bovine encephalitis strain obtained from Drs. Jones and Little.

In 1932, TenBroeck, studying a fowl disease characterized by massive necrosis of the myocardium, which was appearing sporadically in the stock chickens at Princeton, isolated an organism with which the disease could be reproduced by intravenous inoculation of normal chickens. In the course of our further study of this disease and of the associated organism, we were led to compare it with the ovine and bovine encephalitis organism, with *B. monocytogenes*, and with the human strains of Burn. Except for the details noted below, these strains, with the exception of *B. monocytogenes*, are indistinguishable. The latter, although similar in every other respect, is immunologically distinct. The description of this group which follows is based on a study of 3 strains from myocarditis in fowl, 3 from bovine encephalitis, 3 from the New Jersey sheep encephalitis, 2 from a case of meningitis in man,<sup>1</sup> and 1 strain of *B. monocytogenes* from the rabbit disease.<sup>2</sup>

### *Morphology and Cultural Appearance*

The members of the group are indistinguishable morphologically, appearing as small rods 1 to  $2\mu$  in length and about  $0.6\mu$  in width. In sections of the heart muscle of fowl dying of the myocardial disease, the bacilli, often appearing directly in the muscle fibres, may be much elongated. They are Gram-positive, although as the culture advances in age this property is lost. When examined at 48 hours they are easily decolorized, perhaps a third of the organisms retaining the dye. In such cultures the Gram stain may show an uneven distribution in individual cells; bipolar staining is common. They are not acid-fast. No spores can be demonstrated.

All the strains exhibit the same type of motility, best seen in a 4 hour dextrose broth culture. In any one field in a hanging drop, a few individuals will be seen actively moving in a tumbling or spiral manner. These may cease and others commence, often with a preliminary violent spinning. By means of the Leifson (7) flagella stain it was possible to demonstrate flagella on all of the strains. 4 to

<sup>1</sup> These strains were kindly supplied by Dr. Burn.

<sup>2</sup> This strain was obtained from Dr. Murray.

18 hour agar slant cultures were used in the manner recommended by Leifson. It was, however, necessary to double the amount of basic fuchsin and to increase the time of staining beyond that stipulated. In those portions of the preparations where isolated organisms could be studied, they were monoflagellate, the flagellum usually located at one pole of the bacillus. However, these structures are apparently easily broken off; the real arrangement may be otherwise.

In a semisolid medium<sup>3</sup> containing 1 per cent dextrose a peculiar type of growth was common to all the strains. Growth along the stab occurred in 24 hours at 37°C., after 48 hours the appearance seen in Fig. 1 developed. Minute colonies surrounded the line of inoculation at varying distances. In some of these cultures there appeared an occasional clouding emanating from the stab line but this had not spread through the entire tube and was not related to any particular depth in the medium.

Non-motile organisms, *B. pullorum* and *B. murisepticus*, were used as controls. They failed to migrate from the point of inoculation in this medium. This is interesting in view of the "test tube brush" appearance which *B. murisepticus* presents in the usual gelatin medium.

On plain agar the colonies are perfectly smooth and when not crowded are about 1 mm. in diameter after 24 hrs. They are almost transparent; by reflected light they have a faint gray color. On blood agar hemolysis occurs, and growth is slightly better. With the exception of one of the sheep strains which grew granularly in broth, all the strains grow with a uniform turbidity.

Growth is scanty, and unless a large inoculum is used it may fail to occur. However, the addition of 1 per cent dextrose to digest or meat infusion broth results in very heavy growth with a slight sediment after 24 hours. A 1 per cent dextrose digest broth was employed in this work. In gelatin at room temperature a line of small discrete colonies develops along the line of the stab.

### *Metabolic Activities*

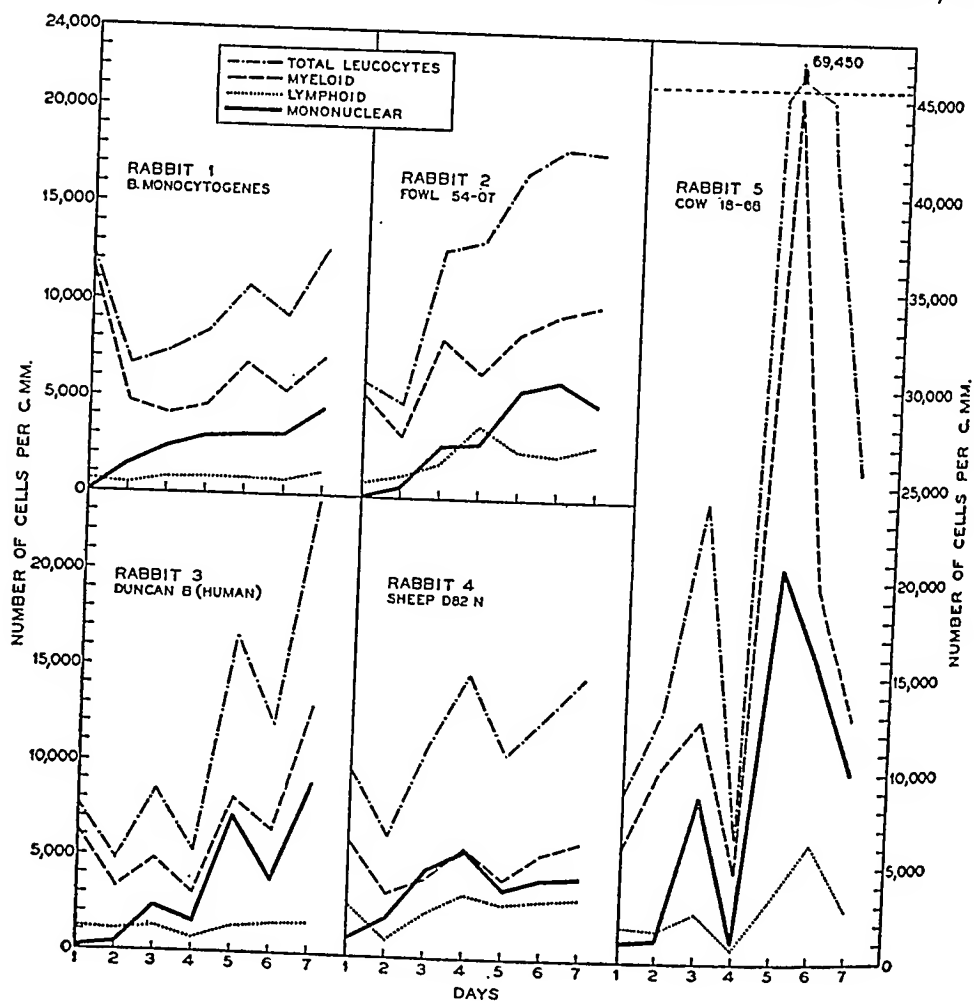
All of the strains ferment dextrose, rhamnose, and salicin promptly; brom-cresol purple becomes yellow in 24 to 48 hours. No gas is produced. With dextrin, soluble starch, and saccharose, fermentation with the formation of acid generally takes place, but much more slowly, 7 to 12 days being required. With maltose, lactose, and glycerol the results are inconstant. In maltose, slight acidity is developed in 4 to 7 days. In lactose only a very slight acidity was produced by some of the strains after 12 days. All of the strains produced slight acid in glycerol after 12 days. No gas was produced. There was no action on mannite, inulin, galactose, xylose, arabinose, or dulcitol.<sup>4</sup>

<sup>3</sup> Agar 0.25 to 0.5 per cent, gelatin 8.0 per cent, dextrose 1 per cent in digest broth. The amount of agar used depends upon the extent to which it has been desiccated. The gelatin facilitates stab inoculation which is done while the medium is cold.

<sup>4</sup> The carbohydrates were autoclaved in distilled water and added to the indicator medium in 0.5 per cent concentration.

Gelatin is not liquefied, nitrates are not reduced, and no  $H_2S$  is formed by any of the strains studied.

All of the members of the group are facultative anaerobes. In a broth medium containing 1 per cent dextrose growth is equally abundant under atmospheric conditions, in a hydrogen jar in the presence of heated platinized asbestos, and



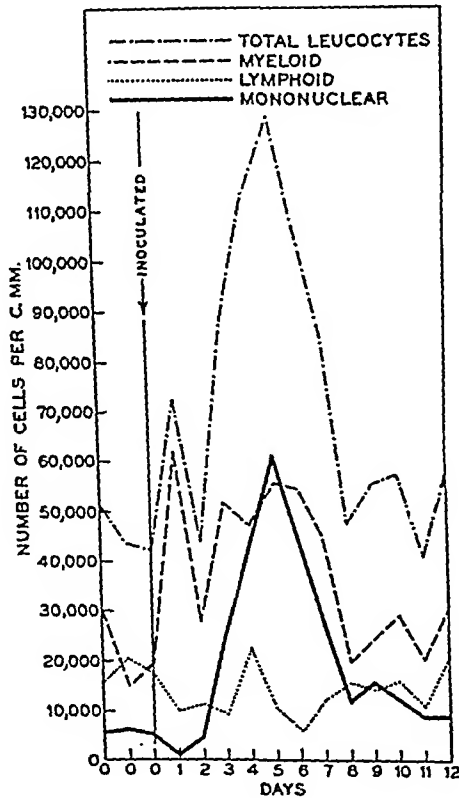
GRAPH 1. Blood response in rabbits to an intravenous injection of 0.2 cc. 24 hour dextrose digest broth culture. The strain used is indicated in each case.

under a vaseline seal in the presence of 1:500 cysteine. Control tubes of broth tinged with methylene blue placed in the hydrogen jar were completely decolorized in the course of 12 hours. Methylene blue in the presence of 1:500 cysteine under a vaseline seal was bleached in less than 5 minutes. Like many facultative anaerobes, anaerobic growth in the absence of carbohydrate was less free than

when aerobic conditions were maintained. Filtrates of 72 hour cultures of the fowl strain, with or without carbohydrate, were not toxic for mice, nor were filtrates of vaseline-sealed cysteine cultures.

### Blood Response

In view of Murray's observation on the increase of the monocytic elements in the blood of rabbits intravenously infected with *B. mono-*



GRAPH 2. Blood response in a chicken to 0.15 cc. 24 hour dextrose digest broth culture of the fowl strain.

*cytogenes* the other strains were tested for this property. Murray comments on the occasional difficulty of classification which some of the cells present when stained by the Leishman method. In our rabbit blood films, stained by Wright, or Wright-Giemsa, so many of the mononuclear cells defied accurate classification that it was found

necessary to employ the supravital stain of Sabin (8).<sup>5</sup> As can be seen from Graph 1, a marked mononucleosis results in 3 to 5 days after the intravenous injection of the organisms. There is usually an increase in the myeloid elements; the lymphocytes are not significantly altered. Although the figures presented do not indicate the relationship between large and small lymphocytes, the large lymphocytes in our counts were relatively rare. It is possible that Murray's high percentages of large lymphocytes were due to atypical monocytes. Individual rabbits vary in their response to the same strain; for this reason Graph 1 is not to be construed as a comparison of the monocytogenic properties of the various strains, but rather as an indication that all the strains possess this property. Guinea pigs develop a mononucleosis as well as rabbits, but to a less marked degree. The monocyte response in chickens intravenously inoculated with the fowl strain is quite constant. Graph 2 represents a typical case. In view of the difficulty of obtaining accurate total leucocyte counts in fowl the figures must be regarded as only approximate.

### *Immunological Relationship*

6 strains were used in determining the serological relationships in the group. They included 2 ovine strains, a bovine, and a fowl strain, a human strain of Burn, and *B. monocytogenes*. Rabbits were immunized against the 6 strains by weekly injections of living cultures.

The first two injections, 0.1 and 0.2 cc. of 24 hour dextrose digest broth culture were given subcutaneously. The subsequent injections were intravenous, beginning at 0.1 cc. and increasing to 1.0 cc. over a period of 5 weeks. 2 weeks after the last injection the rabbits were bled and serum was collected. The antigen employed for agglutination was a 24 hour 1 per cent dextrose digest broth culture adjusted to pH 7.2 and diluted with 5 volumes of salt solution. This neutralization is necessary because of the instability of the organisms in the acid fermentation products from dextrose. Agglutination was carried out for 2 hours at 37°C. and overnight in the refrigerator.

Normal sera bring about a certain amount of agglutination. In the case of horse and chicken serum this is striking. 130 chicken sera, regardless of the age or health of the bird, showed an average titer of 1:160 against the fowl strain. Titers of 1:320 and 1:640 were not infrequent. Four human sera gave an average titer of 1:40; two rabbits showed a titer of 1:80 against the fowl strain.

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<sup>5</sup> I am very much indebted to Dr. Sabin for invaluable advice and instruction in this technic.

Titers from 1:2000 to 1:10,000 were obtained with the immune sera prepared as described above. It was found that the fowl, human, bovine, and ovine strains agglutinated in any of the sera prepared against these strains, with only insignificant differences in titer. *B. monocytogenes*, however, showed no agglutination above the normal serum level in these sera. A serum prepared against *B. monocytogenes* which had an homologous titer of 1:10,000 gave no agglutination with the other strains. The cross-agglutinations are presented in Table I.

Reciprocal absorptions carried out with the five mutually agglutinable strains showed that, with the possible exception of the human and cattle cultures, they are not antigenically identical. As would be

TABLE I  
Cross-Agglutinations

| Organism tested               | Titer limit in serum of rabbit immunized against |             |               |                |                |                         |
|-------------------------------|--|-------------|---------------|----------------|----------------|-------------------------|
|                               | Human strain                                     | Fowl strain | Cattle strain | Sheep A strain | Sheep B strain | <i>B. monocytogenes</i> |
| Human strain.....             | 1:5000   | 1:2000      | 1:1000        | 1:2000         | 1:5000         | 1:160                   |
| Fowl strain.....              | 1:2000   | 1:5000      | 1:2000        | 1:2000         | 1:5000         | 1:160                   |
| Cattle strain.....            | 1:5000   | 1:2000      | 1:2000        | 1:5000         | 1:5000         | 1:160                   |
| Sheep A strain.....           | 1:5000   | 1:5000      | 1:2000        | 1:5000         | 1:5000         | 1:160                   |
| Sheep B strain.....           | 1:5000   | 1:5000      | 1:2000        | 1:5000         | 1:5000         | 1:160                   |
| <i>B. monocytogenes</i> ..... | 1:160  | 1:160       | 1:160         | 1:160          | 1:160          | 1:10,000                |

expected from the absence of cross-agglutination between *B. monocytogenes* and the other strains, no reciprocal absorption took place between *B. monocytogenes* and the fowl strain.

These data indicate that the human, fowl, cattle, and sheep strains are serologically related, and that *B. monocytogenes* differs antigenically from the other strains.

### Variation

In connection with the type of growth noted in semisolid medium (Fig. 1), an explanation was sought for the occasional uniform clouding which appeared along the line of the stab. This seemed especially interesting since it was common to all the strains under observation. By using ordinary culture tubes to which a side arm was attached, it was possible to withdraw a portion of this cloudy type of growth when it occurred sufficiently near the opening of the side arm. This was



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<sup>5</sup> I am very much indebted to Dr. Sabin for invaluable advice and instruction in this technic.

Titers from 1:2000 to 1:10,000 were obtained with the immune sera prepared as described above. It was found that the fowl, human, bovine, and ovine strains agglutinated in any of the sera prepared against these strains, with only insignificant differences in titer. *B. monocytogenes*, however, showed no agglutination above the normal serum level in these sera. A serum prepared against *B. monocytogenes* which had an homologous titer of 1:10,000 gave no agglutination with the other strains. The cross-agglutinations are presented in Table I.

Reciprocal absorptions carried out with the five mutually agglutinable strains showed that, with the possible exception of the human and cattle cultures, they are not antigenically identical. As would be

TABLE I  
Cross-Agglutinations

| Organism tested               | Titer limit in serum of rabbit immunized against |             |               |                |                |                         |
|-------------------------------|--|-------------|---------------|----------------|----------------|-------------------------|
|                               | Human strain                                     | Fowl strain | Cattle strain | Sheep A strain | Sheep B strain | <i>B. monocytogenes</i> |
| Human strain.....             | 1:5000   | 1:2000      | 1:1000        | 1:2000         | 1:5000         | 1:160                   |
| Fowl strain.....              | 1:2000   | 1:5000      | 1:2000        | 1:2000         | 1:5000         | 1:160                   |
| Cattle strain.....            | 1:5000   | 1:2000      | 1:2000        | 1:5000         | 1:5000         | 1:160                   |
| Sheep A strain.....           | 1:5000   | 1:5000      | 1:2000        | 1:5000         | 1:5000         | 1:160                   |
| Sheep B strain.....           | 1:5000   | 1:5000      | 1:2000        | 1:5000         | 1:5000         | 1:160                   |
| <i>B. monocytogenes</i> ..... | 1:160  | 1:160       | 1:160         | 1:160          | 1:160          | 1:10,000                |

expected from the absence of cross-agglutination between *B. monocytogenes* and the other strains, no reciprocal absorption took place between *B. monocytogenes* and the fowl strain.

These data indicate that the human, fowl, cattle, and sheep strains are serologically related, and that *B. monocytogenes* differs antigenically from the other strains.

### Variation

In connection with the type of growth noted in semisolid medium (Fig. 1), an explanation was sought for the occasional uniform clouding which appeared along the line of the stab. This seemed especially interesting since it was common to all the strains under observation. By using ordinary culture tubes to which a side arm was attached, it was possible to withdraw a portion of this cloudy type of growth when it occurred sufficiently near the opening of the side arm. This was

done with the fowl strain. The organism obtained in this way, on reintroduction into semisolid medium, spread out with a uniform clouding all along the stab line, filling the entire tube in the course of 48 hours. Subsequent generations of this strain have shown no tendency to revert to the peculiar type of growth exhibited by the original culture. The variant was found to be slightly less virulent than the original culture when tested in mice. In all other respects no difference could be demonstrated; in rate of growth, colony form, hemolysis, flagella, or agglutination.

In studying this variation it was found that in the tubes of uninoculated semisolid medium methylene blue was reduced with the exception of the topmost centimeter. For this reason the growth of the two forms was compared on the surface of plates using the same medium. Under such aerobic conditions the two forms exhibited a very similar type of growth, both spreading through the medium about the streak-line with a uniform clouding. However, similarly inoculated plates incubated in the hydrogen jar showed that under anaerobic conditions the original strain migrated out from the streak-line practically not at all, whereas the variant produced a uniform clouding. Non-motile control organisms did not migrate from their point of introduction in this medium. A similar variant was isolated from a culture of *B. monocytogenes*. Its virulence has not been determined. One might expect to obtain these variants from all the strains since they showed the same appearance in semisolid medium.

From this it seems reasonable to assume that the motility of the strains in question is dependent on a certain amount of oxygen, but that occasionally variants arise which are capable of movement under anaerobic conditions.

### *Pathology*

In addition to the pathologic changes noted by the authors cited above, it should be pointed out that in the natural disease in chickens, and in chickens, rabbits, or guinea pigs inoculated intravenously with cultures of the fowl strain, one of the most striking changes seen at autopsy is a massive necrosis of the myocardium. There may be very little normal cardiac muscle remaining. There is an associated pericarditis often with large amounts of pericardial fluid or exudate, and congestion of the liver and spleen. This picture is not seen in the

artificially infected animals unless the dosage has been so regulated that a lapse of 5 to 7 days occurs before death. It has never been seen in mice. Although the different strains vary somewhat in virulence, the same involvement of the heart muscle forms a major part of the pathological picture. Figs. 3, 4, and 5 show typical hearts from animals succumbing to the infection. In Gram-Weigert stained sections of the heart, many of the organisms are inside the muscle fibrils, and arranged parallel to them as though extension of the lesion were occurring by way of the muscle tissue (see Fig. 2). In rabbits, cardiac irregularity and signs of cardiac insufficiency have been noted. None of the animals dying of these generalized artificial infections exhibited clinical signs directly referable to the central nervous system. Although the number of rabbits and guinea pigs examined histologically for central nervous system lesions was inadequate, only those animals overwhelmed by the infection showed a slight perivascular cuffing or meningitis.

#### DISCUSSION

It should be noted that of the many strains in this group of organisms, all but one were found to be immunologically related and that one, *L. monocytogenes*, was the one example not indigenous to this country. The antigenic relationship of the New Zealand, South African, and West Australian strains should be of great interest, and might give a clue to the epidemiology of this ubiquitous organism. There can be little doubt that Murray's *L. monocytogenes* belongs to the same general group as the American strains since it possesses in common with these strains an unique combination of properties. The rabbit and gerbille strains already are classified in Bergey's Manual under a new genus, *Listerella*. It would be possible to include the other strains in this genus. It might be suggested that *monocytogenes* as a specific name for one of the members of the group, or genus, is inappropriate since the monocytogenic property is shared by all the members so far examined.

Since this material was prepared for publication, Burn<sup>6</sup> has reported further observations on the human strain. He finds it culturally identical with the rabbit strain (*Listerella monocytogenes*), and in addi-

<sup>6</sup> Reported at the meetings of the Society of Pathologists and Bacteriologists, New York, Apr. 18, 1935.

tion has obtained a mononucleosis in rabbits infected with his organism. He also comments on necrotic heart lesions in guinea pigs.

#### SUMMARY

From meningitis in man, encephalitis in cattle and sheep, a myocardial infection in fowl, and a generalized infection in rabbits, different observers have isolated Gram-positive organisms which are closely related. Their cultural and serological properties are described. When injected intravenously into chickens, rabbits, or guinea pigs there is an unusual blood response, the monocytes being markedly increased. The organisms tend to localize in the myocardium with resulting necrosis.

The author wishes to express his gratitude to Mr. Charles Gordon for technical assistance.

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#### EXPLANATION OF PLATES

##### PLATE 11

FIG. 1. Semisolid medium, 48 hours incubation. (a) Fowl strain. (b) *B. monocytogenes*.

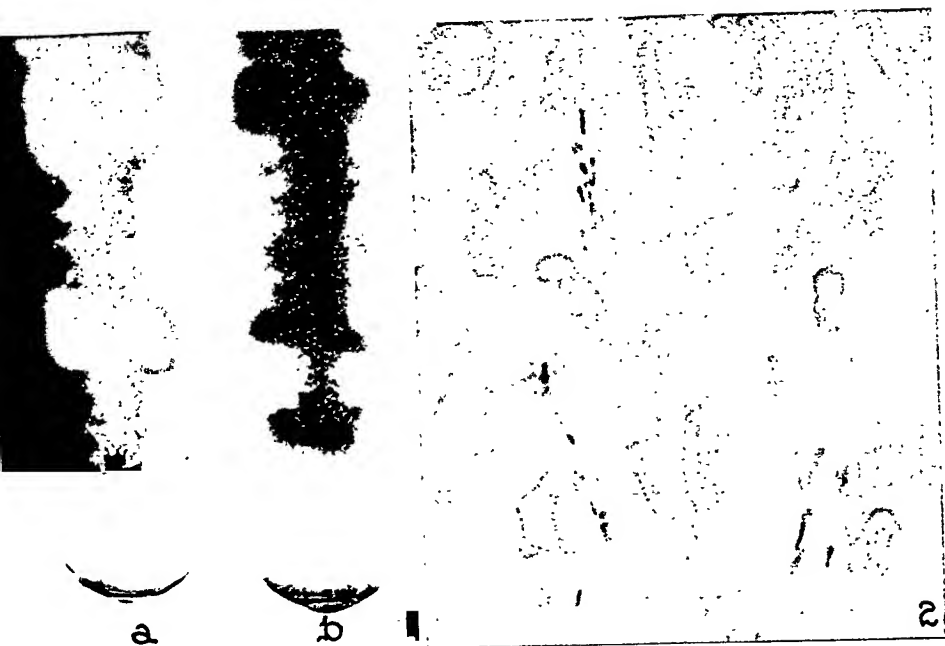
FIG. 2. Guinea pig infected with fowl strain. Heart muscle. Gram-Weigert stain.  $\times 2000$ .

FIG. 3. Chicken heart. Natural infection.

##### PLATE 12

FIG. 4. Rabbit heart. Received 0.2 cc. 24 hour broth culture of the fowl strain. Survived 10 days.

FIG. 5. Guinea pig hearts. Each received 0.05 cc. 24 hour broth culture. Survived from 6 to 12 days. (a) *B. monocytogenes*. (b) Human strain. (c) Fowl strain.



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Photographed by J. A. Carlile

(Seastone: Pathogenic organisms of genus *Listerella*)





Photographed by J. A. Carlile

(Seastone: Pathogenic organisms of genus *Listerella*)





# THE INFLUENCE OF LATENT SYPHILITIC INFECTION ON THE REACTION OF THE RABBIT TO THE BROWN-PEARCE TUMOR

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The problem of the relation between constitution and susceptibility to disease has been studied in this laboratory through two main avenues of approach. The first has been to observe the influence on the host's reaction to disease of various environmental conditions, for example sunlight, ultraviolet radiation, darkness, and meteorological states, of operative procedures and of concomitant infections. The second approach has been from the standpoint of the genetic constitution of the host through an intensive study of breed characteristics and hereditary abnormalities. One technique which has been utilized for the latter purpose has involved observations on the reaction to disease-producing agents of pure bred rabbits of varying lineage and of known quality. Investigations along these lines have demonstrated that the differences which are represented by varying breed characters are associated with distinctive differences in the response to infection with *Treponema pallidum* (1) and to inoculation with a transplantable neoplasm (2). Thus with respect to experimental syphilis, the Havana and Dutch rabbits were found to be relatively resistant while English, Rex, and Himalayan rabbits were relatively susceptible; with regard to the Brown-Pearce tumor, Havana and Himalayan animals were relatively resistant, and Dutch, Rex, and English relatively susceptible.

With this information as a background, a study was made of the effect of an acquired immunity to *Tr. pallidum* on the rabbit's susceptibility to the Brown-Pearce tumor. The experiments which were conducted in order to elucidate this problem form the basis of the present report.

*Material and Methods*

The results are based on an analysis of the reaction to inoculation with the Brown-Pearce tumor of 50 standard bred rabbits with latent syphilitic infection. The animals were all young adult males and were distributed according to breed as follows: Himalayan 15, English 7, Dutch 13, Rex 5, and Havana 10. All animals were housed in individual cages in a well lighted, well ventilated room and were fed a uniform diet composed of a mixture of grains and grain products with alfalfa, mineral salts, and a molasses binder. This was supplemented with hay and a free supply of water.

Intratesticular inoculations with syphilitic material were first made, and the course of the experimental disease followed by frequent clinical examinations. After the lapse of a 4½ to 7 months period, the animals were reinoculated, also intratesticularly, with the Brown-Pearce tumor. The selection of animals for the second inoculation was determined by the complete healing of all lesions and by the presence of clinically normal testicular tissue; the testicle chosen for the tumor injection either had not been involved in the syphilitic process, or if it had, showed healed and regenerated testicular substance. The dates of inoculation with the syphilitic material and of reinoculation with the Brown-Pearce tumor are shown in Table I.

*Inoculation with Tr. pallidum and Estimation of the Severity of the Disease.*—The Nichols strain of *Tr. pallidum* was employed throughout. All the animals were inoculated into one testicle with 0.3 cc. of a saline emulsion prepared from an actively developing testicular lesion and containing from two to six organisms per dark field. Careful clinical examinations were made several times each week for 3 months after inoculation; thereafter, the clinical examinations were less frequent. Particular attention was directed to the presence or absence and time of appearance of the primary orchitis, critical edema, and metastatic orchitis, and the presence or absence, number, and time of appearance of metastatic lesions of the skin, bones and periosteum, and eyes. An estimate of the relative severity of the disease in any particular animal was made by a scheme of percentages which varied with the quantitative occurrence of primary and metastatic lesions. The clinical history of each animal for the 3 months period after inoculation was reviewed and groupings in terms of syphilis morbidity were made according to the following scale: Animals which during the entire course had developed a primary orchitis alone and no other lesion, 10 per cent; animals which developed a primary and a metastatic orchitis and no other lesions, 33 per cent; animals which during the 3 months period had developed both a primary and a metastatic orchitis, together with one to two generalized lesions, 50 per cent; animals which had developed both a primary and a metastatic orchitis and in addition three to five generalized lesions, 67 per cent; and finally, animals which during the observation period of 3 months from inoculation had developed both a primary and a metastatic orchitis, together with six or more generalized lesions, 100 per cent. It should be emphasized that this method does not take into consideration the time of appearance of

a lesion, its persistence, or its qualitative difference from other lesions, and is not recommended for ascertaining fine distinctions between the severity of the disease in different animals. For the present purposes, however, it is entirely satisfactory. Using these estimates of syphilis morbidity, mean values were obtained for each standard bred group under consideration.

*Reinoculation with the Brown-Pearce Tumor and Estimation of the Severity of the Disease.*—After all syphilitic lesions had completely resolved as determined by careful clinical examination, and the state of latency had been established, each animal was reinoculated in one testicle with 0.3 cc. of a saline emulsion of fresh, living Brown-Pearce tumor. The testicle selected for reinoculation either had not been involved in the syphilitic process, or if both testicles had been involved, the better of the two from the point of view of normal testicular substance was injected. The tumor is considered to be an epithelioma (3) and has been carried in this laboratory by animal inoculations for upwards of 140 generations since March, 1921. At the end of a 2 months period after inoculation, the surviving animals were killed by air injection. All animals were autopsied, and special attention was paid to the organ distribution and character of macroscopic tumor growths. Estimations of the severity of the malignant disease were made by a percentage method previously employed by Casey (4) which is based essentially on the number of organs or tissues having tumor growth as determined by autopsy examination; the extent of invasion and the number of tumor nodules in any one organ are not taken into account. Groupings in terms of malignancy were made according to the following scale: Animals which at the end of the 2 months observation showed no tumor at autopsy, 0 per cent; animals with a primary tumor in the inoculated testicle but no metastatic foci, 25 per cent; animals with a primary tumor and one to two metastatic foci, 33 per cent; animals with a primary tumor and three to five metastatic foci not affecting a vital organ, 50 per cent; animals with more than five metastatic foci at autopsy in addition to a primary tumor, or fewer foci but active tumor in a vital organ, 67 per cent; and finally, those which had died of tumor during the observation period, 100 per cent. Using these indices, percentage mean values for mortality were calculated for each breed and for the combined group of 50 animals. In the text these animals have been variously designated experimental, syphilitic, or syphilis-immune group.

*Calculation of Weighted Values for Relative Tumor Malignancy in Normal, Non-Syphilitic Rabbits.*—In a study of the reaction of standard bred rabbits to the Brown-Pearce tumor, Casey (2) described the above method, and by its use determined the mean mortality for several different breeds of rabbits, including those represented in the present investigation; namely, Himalayan, Rex, English, Havana, and Dutch. These values, together with their standard errors and the number of observations on which they are based, are shown in Table II. They give the levels of tumor mortality in normal, non-syphilitic rabbits of the stated breeds, and have been employed as normal control values with which the breed mean observations on the experimental, i.e. syphilitic, groups have been compared. From them also, a weighted mean value, with which was compared the mean value

for the total of 50 observations of the present series, was calculated by the use of the following formula.

$$(1) \quad \frac{\sum (n \times M_{\text{breed}})}{50} = \text{Mean}_{50}$$

where  $n$  represents the number of animals of a particular breed used in the present experiment and  $M_{\text{breed}}$  represents the mean value for the breed as obtained by Casey (2). Furthermore, a new value for the standard error of this weighted mean was determined by an application of the following formula.

$$(2) \quad \sum n(\sigma_{\text{breed}}^2 + M_{\text{breed}}^2) = \Sigma_{50}^2$$

in which  $\sigma_{\text{breed}}$  is the standard deviation and  $M_{\text{breed}}$  the mean value for each of the different breeds in Casey's series. The first formula gave the weighted value for mean tumor malignancy in normal, non-syphilitic rabbits, and this in conjunction with the second gave a new standard error of the mean when the number of observations was equal to 50. An alternate formula for the standard error of the weighted mean was used as a check.

$$(3) \quad \sigma \text{Mean}_{n_2} = \sqrt{\frac{n_1}{n_2}} \cdot \sigma \text{Mean}_{n_1}$$

Here  $n_1$  and  $n_2$  represent the number of observations in Casey's series and the number of observations in the experimental group respectively, and  $\sigma \text{Mean}_{n_1}$  and  $\sigma \text{Mean}_{n_2}$  are the standard errors of the means of  $n_1$  and  $n_2$  observations. This formula is based on the supposition that a second series of  $n_2$  observations would have the same standard deviation as the first series of  $n_1$  observations,<sup>1</sup> and has been used in calculating the standard error of the normal mean values shown in Table II.

The comparisons have been based on mean values in order to eliminate the variations due to individual differences. In all statistical procedures, significance has been attached to values of  $P = 0.01$ ; that is, when the probability of an event occurring by chance was 1 or less than 1 in 100, the result was considered significant. The  $\chi^2$  test is described by Fisher (5).

## RESULTS

The results are presented in Tables I to III and Text-fig. 1. Table I lists the individual observations on each animal and includes the dates

<sup>1</sup> For those who are interested, this formula was obtained as follows:

$$(1) \sigma n_1 = \sigma \text{Mean}_{n_1} \cdot \sqrt{n_1}. \quad (2) \sigma n_2 = \sigma \text{Mean}_{n_2} \cdot \sqrt{n_2}. \quad (3) \text{By hypothesis, } \sigma_{n_2} = \sigma_{n_1}.$$

$$(4) \therefore \sigma \text{Mean}_{n_2} \cdot \sqrt{n_2} = \sigma \text{Mean}_{n_1} \cdot \sqrt{n_1} \text{ and } (5) \therefore \sigma \text{Mean}_{n_2} = \sqrt{\frac{n_1}{n_2}} \cdot \sigma \text{Mean}_{n_1}.$$

TABLE I

*Individual Values for Morbidity from Tr. pallidum Infection and Mortality from the Brown-Pearce Tumor in Standard Bred Rabbits*

| Animal No. | Breed   | Date inoculated with <i>Tr. pallidum</i> | Relative morbidity | Date reinoculated with Brown-Pearce tumor | Relative mortality |
|------------|---------|--|--------------------|---|--------------------|
|            |         |  | <i>per cent</i>    |   | <i>per cent</i>    |
| 546-1      | Dutch   | Apr. 9, 1931                             | 67                 | Oct. 20, 1931                             | 0                  |
| A94B       | "       | Oct. 26                                  | 100                | Mar. 25, 1932                             | 100                |
| A94C       | "       | " 26                                     | 67                 | " 25                                      | 100                |
| A108A      | "       | " 26                                     | 33                 | " 25                                      | 33                 |
| A108B      | "       | " 26                                     | 33                 | " 25                                      | 33                 |
| D20-4      | "       | Mar. 31, 1932                            | 10                 | Oct. 7                                    | 0                  |
| D17        | "       | " 31                                     | 33                 | " 7                                       | 50                 |
| D26-1      | "       | " 31                                     | 33                 | " 7                                       | 0                  |
| D26-2      | "       | " 31                                     | 33                 | " 7                                       | 0                  |
| D23-2      | "       | " 31                                     | 10                 | " 7                                       | 0                  |
| D23-3      | "       | " 31                                     | 50                 | " 7                                       | 25                 |
| D120-1     | "       | Oct. 31, 1933                            | 33                 | Mar. 13, 1934                             | 25                 |
| D85        | "       | " 31                                     | 50                 | " 13                                      | 25                 |
| A66-6      | Havana  | Oct. 26, 1931                            | 100                | Mar. 25, 1932                             | 0                  |
| A66-5      | "       | " 26                                     | 10                 | " 25                                      | 0                  |
| A96A       | "       | " 26                                     | 100                | " 25                                      | 0                  |
| 925A       | "       | " 26                                     | 67                 | " 25                                      | 0                  |
| HA1-1      | "       | Mar. 31, 1932                            | 33                 | Oct. 7                                    | 0                  |
| HA1-2      | "       | " 31                                     | 33                 | " 7                                       | 0                  |
| HA1-3      | "       | " 31                                     | 10                 | " 7                                       | 0                  |
| HA1-5      | "       | " 31                                     | 33                 | " 7                                       | 0                  |
| HA1-6      | "       | " 31                                     | 10                 | " 7                                       | 0                  |
| HA4-7      | "       | " 31                                     | 10                 | " 7                                       | 0                  |
| 561-4      | English | Apr. 9, 1931                             | 100                | Oct. 20, 1931                             | 100                |
| E1-2       | "       | Mar. 31, 1932                            | 50                 | Oct. 7, 1932                              | 67                 |
| E8-3       | "       | " 31                                     | 33                 | " 7                                       | 25                 |
| E8-6       | "       | " 31                                     | 10                 | " 7                                       | 67                 |
| E10        | "       | " 31                                     | 100                | " 7                                       | 25                 |
| E11-2      | "       | " 31                                     | 33                 | " 7                                       | 50                 |
| E10-2      | "       | Oct. 31, 1933                            | 33                 | Mar. 13, 1934                             | 67                 |
| 958A       | Rex     | Oct. 26, 1931                            | 100                | Mar. 25, 1932                             | 100                |
| R106-2     | "       | Oct. 31, 1933                            | 33                 | Mar. 13, 1934                             | 0                  |
| R71        | "       | " 31                                     | 100                | " 13                                      | 50                 |
| R94        | "       | " 31                                     | 50                 | " 13                                      | 0                  |
| R731-1     | "       | " 31                                     | 33                 | " 13                                      | 67                 |

TABLE I—*Concluded*

| Animal No. | Breed     | Date inoculated with <i>Tr. pallidum</i> | Relative morbidity | Date reinoculated with Brown-Pearce tumor | Relative mortality |
|------------|-----------|--|--------------------|---|--------------------|
|            |           |  | <i>per cent</i>    |   | <i>per cent</i>    |
| 939B       | Himalayan | Oct. 26, 1931                            | 100                | Mar. 25, 1932                             | 33                 |
| A24A       | "         | " 26                                     | 33                 | " 25                                      | 0                  |
| 716-3      | "         | " 26                                     | 100                | " 25                                      | 67                 |
| 822A       | "         | " 26                                     | 100                | " 25                                      | 33                 |
| H5-5       | "         | " 26                                     | 67                 | " 25                                      | 0                  |
| H6-1       | "         | Mar. 31, 1932                            | 67                 | Oct. 7                                    | 0                  |
| H6-3       | "         | " 31                                     | 67                 | " 7                                       | 0                  |
| H6-5       | "         | " 31                                     | 100                | " 7                                       | 0                  |
| H7-2       | "         | " 31                                     | 10                 | " 7                                       | 0                  |
| H8-2       | "         | " 31                                     | 33                 | " 7                                       | 0                  |
| H50-1      | "         | " 31                                     | 50                 | " 7                                       | 0                  |
| H50-2      | "         | Oct. 31, 1933                            | 100                | Mar. 13, 1934                             | 0                  |
| H58        | "         | " 31                                     | 100                | " 13                                      | 0                  |
| H49-2      | "         | " 31                                     | 100                | " 13                                      | 0                  |
| H53        | "         | " 31                                     | 100                | " 13                                      | 50                 |

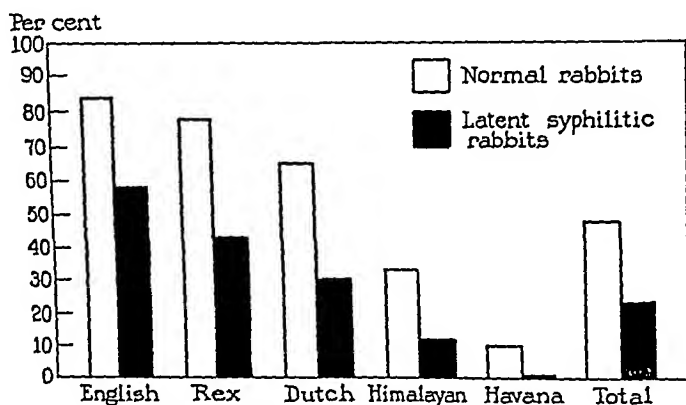
TABLE II

*Relative Mortality from Malignant Neoplasm in Syphilis Animals Compared with Values for Normal Animals*

| Breed          | Syphilitic group |                 | Normal group (Casey (2))<br>Actual values |                 | Normal group<br>Weighted values |                 |
|----------------|------------------|-----------------|---|-----------------|---------------------------------|-----------------|
|                | No. of animals   | Mean            | No. of animals                            | Mean            | No. of animals                  | Mean            |
|                |                  | <i>per cent</i> |   | <i>per cent</i> |                                 | <i>per cent</i> |
| English.....   | 7                | 57.1 ± 10.1     | 25  | 84.0 ± 6.4      | 7                               | 84.0 ± 12.1     |
| Rex.....       | 5                | 43.3 ± 19.4     | 13  | 77.6 ± 6.8      | 5                               | 77.6 ± 10.9     |
| Dutch.....     | 13               | 30.1 ± 9.8      | 18  | 65.3 ± 7.8      | 13                              | 65.3 ± 9.2      |
| Himalayan..... | 15               | 12.2 ± 5.7      | 27  | 33.3 ± 6.6      | 15                              | 33.3 ± 8.9      |
| Havana.....    | 10               | 0 ± 0           | 18  | 11.1 ± 4.8      | 10                              | 11.1 ± 6.5      |
| Total.....     | 50               | 23.8 ± 5.1      | 101                                       | 53.8 ± 4.1      | 50                              | 48.7 ± 5.7      |

of inoculation with *Tr. pallidum* and of reinoculation with the Brown-Pearce tumor, together with values for syphilis morbidity and for tumor mortality as determined by the methods heretofore described. Table II records the observations on the mortality of the syphilitic animals of the present investigation after inoculation with the Brown-

Pearce tumor, together with the mean values for mortality from the Brown-Pearce tumor obtained by Casey (2) in his study on the reaction of normal animals to inoculation with that tumor, and weighted normal mean values derived from Casey's observations, as described in the section on Material and Methods. In this table the observations are presented from the standpoint of the standard breed of rabbit under consideration; namely, the English, Rex, Dutch, Himalayan, and Havana. Text-fig. 1 indicates graphically the mean values for syphilitic animals and the weighted values for normal animals, which are given in Table II.



TEXT-FIG. 1. Mean values for mortality from the Brown-Pearce tumor in latent syphilitic and in normal non-syphilitic rabbits.

Table II and Text-fig. 1 show that the mean value for tumor mortality in the 50 syphilitic animals was  $23.8 \pm 5.1$  per cent. This is significantly lower than the weighted mean normal value of  $48.71 \pm 5.7$  per cent (difference =  $-24.9 \pm 8.0$ ,  $t = 3.11$ ,  $P = 0.01$  —, significant). Also the mean value for each breed was lower in the syphilitic group than the corresponding breed mean value for normal animals.

#### DISCUSSION

Kolle (6), Chesney (7), and others have demonstrated that following a first intratesticular inoculation with *Tr. pallidum*, reinoculation with a homologous strain before the lapse of 60 days produces a new chancre in more than half of the observations, while reinoculation



after a 90 day period rarely results in a chancre. This type of acquired immunity to syphilis reinoculation is operative only in the case of homologous virus. On this basis the animals of the present investigation were all presumed to be immune to homologous virus since a 5 to 7 months period had elapsed between the syphilis and tumor inoculations. It is for this reason that the experimental animals have been referred to as the syphilis-immune group.

These experiments were planned in order to determine whether the immune syphilitic state in the rabbit altered its absolute level of susceptibility to inoculation with a malignant neoplasm. It has been seen that the mean value for tumor mortality in the 50 syphilitic animals was significantly lower than the weighted mean normal value. It had been expected to employ as control groups animals of identical breed inoculated with the same tumor emulsion which the experimental group received. This procedure was, however, not feasible because of the relative scarcity of pure bred rabbits of the required breeds, and for comparative purposes, therefore, the breed mean mortality values obtained by Casey on normal animals have been resorted to.

There is evidence that the tumor with which a large proportion of the experimental or syphilis-immune animals was inoculated, was capable of inducing a highly malignant disease. Twenty-three or 46 per cent of the 50 experimental animals were inoculated with tumor on October 7, 1932, together with three normal non-syphilitic animals. All three normal rabbits died with widespread tumor metastases, but the mortality rate in the experimental animals was 13.5 per cent. A similar lower rate obtained when only the most susceptible of all breeds, the English, comprised the experimental group, for the mean mortality rate in five syphilis-immune English rabbits inoculated on October 7, 1932, was  $46 \pm 10.4$  per cent, while the mortality rate in the three normal animals inoculated on the same date was more than double this value. Furthermore, the neoplasm is known to be most malignant in the fall and spring (8), the seasons of the year when the present tumor inoculations were made. In spite of the fact that seasonal conditions were optimum for the growth and malignancy of the tumor, the mortality rate in all five breeds and in the combined group of 50 animals was lower than normal control values.

It will be recalled that the syphilis-immune group which was inoculated with tumor comprised animals selected because of presumably normal testicular tissue, and that the testicle chosen for reinoculation either had not been involved in the syphilitic process, or showed regenerated testicular substance. It seemed quite possible, however, that even though a testicle had completely healed clinically, an antecedent syphilitic process might have produced in it a local refractoriness to tumor growth. This does not appear to be the case. Considering the English, Rex, and Dutch groups as relatively susceptible to the tumor, of 22 animals of these breeds which had developed a primary and a metastatic syphilitic orchitis, the tumor grew at the site of injection in 17 or 77.3 per cent of instances, and of these 17 animals, metastases to distant sites occurred in 12 or 70.6 per cent. Thus tumor grew in a large proportion of the healed syphilitic testicles of susceptible animals. Conversely, tumor did not grow in a large majority of testicles which had never presented clinical evidence of a syphilitic process, for of seven animals which developed only a primary orchitis, tumor inoculation into the unaffected testicle on October 7, 1932, with material which has been shown to be highly malignant in normal animals, produced a growth in only one instance. From this evidence, it appears that the local testicular reaction to *Tr. pallidum* could not account for the observed difference between the mortality rates in syphilitic as compared with non-syphilitic rabbits.

Additive information which makes this difference of increased significance is the fact that each of the five breed mean values for tumor mortality in the syphilis-immune group was lower than the mean values for normal animals of corresponding breeds. Not only was this the case, but the establishment of an immunity to syphilis produced no alteration in the relative susceptibility of different breeds of rabbits inoculated with the tumor. Table II shows that the mortality rates from the Brown-Pearce tumor in the groups of standard bred normal (non-syphilitic) rabbits of Casey's series ranged from 84.0 per cent for the English to 11.1 per cent for the Havana, with the Rex, Dutch, and Himalayan groups occupying intermediate positions between these extremes at levels of 77.6, 65.3, and 33.3 per cent respectively. Using the same technique of inoculation, and the same criteria for the determination of malignancy, the tumor mortality in

the syphilis-immune animals ranged according to breed from a high mean level of 57.1 per cent for the English, to a low of 0 per cent for the Havana, with intermediate positions of 43.3, 30.1, and 12.2 per cent occupied by the Rex, Dutch, and Himalayan breeds respectively. Thus the relative positions of the five breeds under consideration on a scale of increasing susceptibility to the Brown-Pearce tumor was the same in the experimental group of syphilis-immune animals as it was in the normal control groups, that is, Havana, Himalayan, Dutch, Rex, and English. This complete correspondence is shown in Text-fig. 1.

In order to determine whether the different breeds were homogeneous with respect to the mortality ratios in the syphilitic and non-syphilitic groups, or otherwise stated, whether the ratio of the mortality in the syphilitic animals to the mortality in the non-syphilitic animals was the same in each breed, Table III was constructed, and a value for  $\chi^2$  equal to 9.72 was obtained. This value would be exceeded by random sampling five times in 100 such experiments, and is probably significant ( $P = 0.05$ ). From Table III it is seen that the principal discrepancy between the observed and the expected values lies in the Havana values ( $S \frac{\chi^2}{n} = 5.78$ ), the actual result in the experimental group being much lower than expected. However, even where a discrepancy between observed and expected values did exist, as in the case of the Havana breed, the difference was in the direction of increased resistance to tumor in the syphilis-immune group. Excluding the Havanas, a new  $\chi^2$  determination showed no significant heterogeneity between the breeds with respect to the ratio of the mortality in syphilitic animals to the mortality in non-syphilitic animals ( $\chi^2 = 3.65$ ,  $n = 3$ ,  $P = 0.30$ , not significant); in other words, the breeds were significantly homogeneous as regards this ratio. This homogeneity is of importance since it demonstrates that the factors which produced increased breed resistance to the tumor inoculation were operative on each breed with equal effectiveness.

The genesis of this increased resistance to tumor in syphilis-immune animals is at present a matter of conjecture. Bashford's original work (9) which is now well established indicates that a definite resistance to tumor can be induced by antecedent inoculations with normal blood,

and others have shown that in the same manner resistance to tumor can be produced by preliminary injections with normal liver, testes, or embryo. It is the consensus of opinion that this type of resistance can only be evoked by the use of living, homologous cells (10).

It is, of course, evident that living homologous cells were present in the original inoculum of syphilitic material employed in this investigation, and it is conceivable that the increased resistance to subsequent

TABLE III

*Relative Mortality in Groups of Standard Bred Rabbits Following Inoculation with the Brown-Pearce Tumor. Observed and Expected Mean Values in Syphilitic and Normal Groups*

*Observed Values (Per Cent)*

| Group           | English | Rex   | Dutch | Himalayan | Havana | Total |
|-----------------|---------|-------|-------|-----------|--------|-------|
| Syphilitic..... | 57.1    | 43.3  | 30.1  | 12.2      | 0      | 142.7 |
| Normal.....     | 84.0    | 77.6  | 65.3  | 33.3      | 11.1   | 271.3 |
| Total.....      | 141.1   | 120.9 | 95.4  | 45.5      | 11.1   | 414.0 |

*Expected Values (Per Cent)*

| Group                                 | English | Rex   | Dutch | Himalayan | Havana | Total |
|---------------------------------------|---------|-------|-------|-----------|--------|-------|
| Syphilitic.....                       | 48.6    | 41.7  | 32.9  | 15.7      | 3.8    | 142.7 |
| Normal.....                           | 92.5    | 79.2  | 62.5  | 29.8      | 7.3    | 271.3 |
| Total.....                            | 141.1   | 120.9 | 95.4  | 45.5      | 11.1   | 414.0 |
| $S\left(\frac{x^2}{m}\right)^*$ ..... | 2.28    | 0.10  | 0.37  | 1.19      | 5.78   | 9.72  |

\*  $S = \text{sum}$ ;  $m = \text{expected value}$ ;  $x = \text{difference between observed and expected value}$ .

inoculation with tumor was due to the same set of factors that produces resistance to tumor after the injection of living normal cells. What part the spirochete and the syphilitic phenomena of tissue reactivity to the spirochete played in the production of the resistance to the tumor is not known. If the resistance were primarily due to the original single injection of living cells, then it was built up and persisted over a period of  $4\frac{1}{2}$  months and more, that is, the interval be-

tween the original injection of syphilitic material and the reinoculation with tumor, and this period is longer than any previously recorded. All the animals were in excellent physical condition at the time of the tumor transplants, and they presented no clinical evidence of syphilis or other disease, so that the increased resistance to tumor cannot be explained on the basis of a sickly host supplying a poor soil for the nurturing of tumor cells. In this connection, it is of interest to recall the work of Rous (11) who found that intercurrent illness of the host may check the further development of a tumor nodule, and may even cause its retrogression.

As is well known, during the period of latency in the untreated syphilitic rabbit, the spirochete resides in lymphoid tissue from which it can be recovered quite readily by subinoculations into normal animals. The presence of the spirochete in the lymphoid tissue of the syphilis-immune group of the present investigation may have been a constant stimulus to the building up of resistance to tumor inoculation, in exactly the same way that repeated injections of fresh living cells produce resistance. In this connection it would be of great interest to determine whether sterilization of the spirochetes by vigorous anti-syphilitic treatment after the establishment of an active infection would alter in any way the production of a tumor resistant state.

The method employed in the evaluation of the severity of the syphilitic disease was based on experience with large numbers of animals. Its use as an estimate of syphilis morbidity has been found to agree with other methods previously followed. Thus a previous analysis of the reaction of standard bred rabbits to experimental syphilis took into careful consideration all the factors of incidence and time of occurrence of various manifestations of the disease, together with the actual and relative focal distribution of generalized lesions, the duration of the period of disease activity, the location and destructiveness of generalized lesions, and the state of lesion activity at the end of a 3 month observation period. From this analysis it was concluded that the relative positions of five breeds of rabbits on a scale of increasing resistance to syphilitic infection was Himalayan, Rex, English, Dutch, and Havana. The first three breeds were considered to be susceptible and the last two resistant. The present analysis on the basis of arbitrarily designated percentages for each animal accord-

ing to the presence or absence of primary and generalized lesions, gave the following mean values: Himalayan 75.1, Rex 63.3, English 51.4, Dutch 42.6, and Havana 40.7. The relative degree of susceptibility to syphilis for different breeds was thus identical with that disclosed by the earlier more precise analysis.

Breed mean values for mortality from the Brown-Pearce tumor in normal non-syphilitic animals are shown in Table II. It will be seen that the Havana and Himalayan breeds are relatively resistant and the Dutch, Rex, and English relatively susceptible. Three of the five breeds under consideration thus showed striking correspondence between resistance to tumor and resistance to syphilis. The English and Rex rabbits were both highly susceptible to syphilis and also to tumor, while the Havana group was the most resistant of all the five breeds to the two diseases. There was a very slight discrepancy in the case of the Dutch since this group was moderately resistant to syphilitic infection and only slightly susceptible to the tumor. However, the greatest disagreement is seen to lie in the values for the Himalayan breed, which showed this group to be highly susceptible to *Tr. pallidum* and very resistant to the tumor. At first glance this would seem to indicate that the non-specific host factors which are responsible for resistance to one disease do not necessarily induce a state of resistance to a second disease. Many isolated observations, however, of which two striking examples have recently been observed, seem to reconcile the apparent discrepancy between the Himalayan's reaction to the two diseases.

In a previous report on the reaction of standard bred rabbits to experimental syphilis (1), the mean interval between inoculation and the appearance of the first and last generalized lesion was longer in the Himalayan group than that observed in Havana, Dutch, English, and Rex groups. This indicates that the defence reaction of the Himalayan animals was more successful than that of other breeds in suppressing the appearance of generalized lesions, but that this reaction was only partially effective since it merely postponed their development. From the standpoint of effectiveness in delaying the appearance of generalized disease manifestations, the Himalayan breed can be considered to be resistant to syphilis. Recently, we have observed the same phenomenon of delay in animals of this breed as regards their reaction to tumor.

Two normal animals of Himalayan lineage showed small tumor nodules in the inoculated testicle 2 months after inoculation. For some time thereafter, these primary tumors presented no evidence of growth, but they then began to increase in size until 9 and 7½ months after inoculation they had attained enormous proportions, and still showed signs of growth. The first animal was found dead 10 months after inoculation, with a large mass of actively growing tumor in the pelvis, and metastases to the pelvis of both kidneys, the opposite testicle, the bladder, the muscles of the abdominal wall and thigh and the spinal cord. In spite of the extensive metastases, the animal was in a fair state of nutrition, moderately large masses of fat being present in the perirenal spaces and in the omentum. Similarly, the second animal, also of Himalayan lineage, which was killed 8½ months after inoculation because of weakness and loss of weight, showed a large pelvic mass consisting of actively growing tumor, and a chain of metastatic tumor in the retroperitoneal lymph nodes. Evidence that the tumor in this animal was living and capable of growth 8½ months after the original transplant was obtained by successful takes in each of five normal animals.

The reaction in both of these cases was evidently similar to that observed in syphilitic Himalayans, for the defence forces of these two animals were sufficiently powerful to suppress the growth of tumor for a considerable period of time after which the defence mechanism became less efficient and the tumor began to grow. On this basis, the only real discrepancy, that noted in the Himalayan group, between breed resistance to syphilis and breed resistance to tumor is reconciled, or at least less accentuated than it appeared to be on the basis of tests which placed a 2 months limitation on the observation period for tumor inoculations. Evidently, the time was too short to permit an accurate estimation of the end-reaction. The fact remains that the Himalayan presents a peculiarity of response to both syphilis and tumor inoculation which, so far, has not been encountered in other breeds and is worthy of note as a racial characteristic which should be taken into account in experiments involving the use of animals of this breed.

#### SUMMARY

The reaction of the latent syphilitic rabbit to inoculation with the Brown-Pearce tumor was studied in 50 standard bred rabbits representing five breeds. The mean tumor mortality rate in the combined group was found to be significantly lower than a weighted control value for normal non-syphilitic animals, and the mean tumor mortality rate for each of the five breeds studied was lower in the syphilitic group

than the corresponding breed mean value for normal animals. Moreover, the relative resistance of different breeds to the Brown-Pearce tumor was not altered by the latent syphilitic infection. Certain factors which might have contributed to the development of a tumor resistant state in the syphilitic group were discussed, and evidence was presented which demonstrates that as regards the breeds under consideration, there exists a high correlation between breed resistance to *Tr. pallidum* infection and breed resistance to the Brown-Pearce tumor.

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# SPREADING PROPERTY OF AZOPROTEINS IN THE DERMIS

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PLATE 13

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The presence in testicle extracts of a factor enhancing the lesions produced by infectious agents was demonstrated by Duran-Reynals (1). Subsequently McClean (2) and Hoffman and Duran-Reynals (3) reported that testicle extract had the power to diffuse through certain tissues, especially the connective tissue of the skin. Extracts from other mammalian organs were found to have these properties, though to a lesser degree (4, 5). Attempts by several workers to demonstrate similar properties with a variety of chemicals and with natural products from animal tissues were unsuccessful (6).

In the course of a study dealing with the effect of tryptic digestion on the spreading power of testicle extract we were led to test the action of certain diazo compounds on skin permeability. These compounds proved to be very active diffusing agents. The results obtained with sulfanilic acid derivatives have been reported in a preliminary note (7).

The properties of the diazo and azo compounds have been studied further and the specificity of the active chemical group has been investigated.

## *Properties of p-Diazobenzenesulfonic Acid and Its Effect on Skin Permeability*

Sulfanilic acid, from which the diazo compound is prepared, has no apparent action on dermal permeability. Solutions of sulfanilic acid at various concentrations, neutralized with sodium hydroxide and injected intradermally with India ink as indicator, leave a wheal of diffusion which does not differ from that produced by water or by isotonic salt solution.

The diazo compound, namely *p*-diazobenzenesulfonic acid, which was prepared by treating sulfanilic acid with nitrous acid is a white, crystalline substance, sparingly soluble in cold water. The compound is known to be unstable, decomposing with loss of nitrogen, especially in alkaline solution, and in dry form it may decompose with deflagration. In presence of acid, the product is fairly stable when moist and kept at ice box temperature.

In contrast to sulfanilic acid, the diazo compound has a striking effect on skin permeability. The inoculation of 0.5 cc. of a 1.5 per cent solution was followed by abrupt spread, indicated by the rapid fading of the bleb of injection and the widespread diffusion of the ink particles. The maximum spread, about 20 sq. cm., was reached in a few hours. After 24 hours there was at the site of inoculation a circular area of about 3 cm. in diameter, deep blue and surrounded by a more faintly colored zone of spread. Larger areas of spread could not be obtained by the use of more concentrated solutions. The compound proved to have some irritating effect on the tissues, as indicated by the development of redness and discrete hemorrhages in the skin treated.

Tested for its effect *in vitro* the diazo compound proved highly anti-septic. Suspensions of *Staphylococcus aureus* and *albus*, *B. coli*, and *B. prodigiosus* were mixed with equal volumes of a neutral solution of the diazonium salt. 2 or more minutes of contact with the chemical completely suppressed the growth of subcultures of the treated organisms on agar and in broth.<sup>1</sup> The mixtures as well as the freshly prepared solutions of the diazo compound gave a positive test with the potassium iodide-starch indicator. This was the result of a slow decomposition of the product which was accompanied by effervescence and liberation of nitrogen. The spontaneous decomposition of the product was accelerated by boiling the solution or by the addition of alkali. Both methods resulted in a stable product which had lost its toxicity as well as its oxidizing property, but in this case also much of its spreading power. According to these observations, the iodide-starch indicator was used as an index of the toxicity of solutions of the diazo compound.

<sup>1</sup> I am indebted to Dr. F. Duran-Reynals for the performance of these tests.

*Preparation and Properties of Diazobenzenesulfonic Acid Coupled with Substances of Small Molecular Weight*

Coupling of the diazo compound with tyrosine, histidine, sulfanilic acid, and a number of other substances of relatively small molecular weight was carried out for the purpose of obtaining a stable azo compound. The combination of these substances in molecular proportions with a 1 per cent solution of the diazo compound resulted in products colored orange to deep red. The rubefacient effect on the skin and the bactericidal property of the free diazo compound were reduced by coupling, but the type and extent of spread obtained on intradermal inoculation were very similar to those of the free diazide.

*p*-arsanilic acid as source of the diazo compound was also substituted for sulfanilic acid and its diazo derivative coupled with tyrosine. This product was precipitated from solutions by means of glacial acetic acid and washed several times with alcohol and ether. A 1.4 per cent solution of the dry tyrosine bis-azobenzenearsanilic acid was made in water and in horse serum. The spread of India ink resulting from intradermal inoculation of these solutions was of the same type and general extent as that obtained with other diazo derivatives: on the average 14.4 sq. cm. as compared with 5.7 sq. cm. of spread induced by Ringer's solution, or by normal horse serum.

*Preparation and Properties of Azoproteins*

*Azoserum.*<sup>2</sup>—The failure to obtain a widespread diffusion with diazonium salts or with azo derivatives of relatively small molecular weight was tentatively explained by the great diffusibility of the products. It was assumed that the effect of the substance would be but slight if it were taken up rapidly into the blood and carried away.<sup>3</sup> Therefore, better results were sought by combining the diazobenzene-sulfonic acid to larger molecules. Coupling with sera of various origins, egg albumin, casein, and gelatin was attempted. On skin inoculation the resulting azoprotein gave a spread similar in type and

<sup>2</sup> The term "azoserum" has been adopted in this work as an abbreviation for "azoprotein prepared from serum proteins."

<sup>3</sup> Since later work with azoproteins proved satisfactory, no attempts were made to confirm this hypothesis by appropriate experiments.

extent to that commonly effected by testicle extract. In the following work, horse serum was mainly used for the preparation of the azo-protein. However, a 3 per cent egg albumin could be used with equally good results.

*Preparation of the Diazo Compound.*—1.045 gm. sulfanilic acid (5 millimoles  $p\text{-(NH}_2\text{)}.C_6H_4(SO_3H)$  +  $2H_2O$ —molecular weight, 209.14) was suspended in 10 cc.  $N$  HCl. The reaction must be performed at low temperature—around  $0^\circ C$ . 5 cc.  $N$   $NaNO_2$  are added slowly with continuous stirring, and the progress of the reaction followed with KI-starch paper as indicator. Absence of free  $HNO_2$  is indicated by a negative reaction: the diazo compound itself oxidizes the starch paper, but with a certain delay, and one can easily differentiate between this and the effect of nitrous acid.

The diazonium salt which was formed settles out as a white crystalline material. About 2.4 cc. of this moist compound was collected by centrifugation and the acid supernatant fluid discarded. The material was washed rapidly in the centrifuge tube by suspending the precipitate in 12 cc. of ice cold distilled water. The washing was repeated twice in order to rid the reagent of excess hydrochloric acid. The last washing should be neutral to Congo red. After the last centrifugation about 1.2 cc. of the crystalline substance was left in the test tube, or roughly one-half of the starting material, corresponding approximately to 2.5 millimoles of the diazo compound.

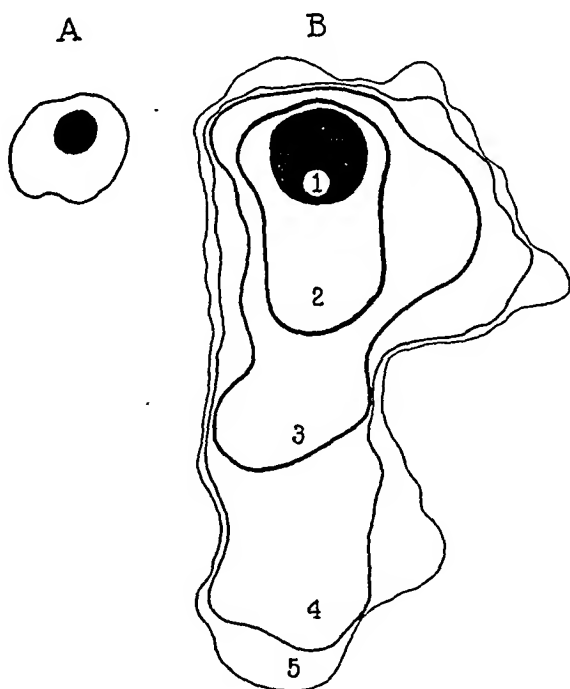
*Coupling with Horse Serum.*—Coupling was accomplished by adding 3.5 cc.  $N$   $NaOH$  to 10 cc. horse serum, to give a pH of at least 9.0, and by adding to this alkaline mixture the washed diazo compound suspended in a few cubic centimeters of cold distilled water. This part of the reaction was also performed at a temperature near to  $0^\circ C$ . Coupling was indicated by a deep red color which developed instantly. The solution was made up to a volume of 30 cc. with distilled water and the preparation neutralized by means of normal acetic acid. This method is not quite quantitative for the azo reaction, but has given the best results as regards the desired type and extent of spread.

Azoserum solutions prepared according to the foregoing method had a total solids content of about 55 mg. per cc., calculated on the basis of 2.5 millimoles diazide per 30 cc. After dialysis the solution was brought back to its original volume and the dry weight of the azoserum solution found to be 32 mg. per cc.

*Diffusion of Azoserum in the Rabbit Skin.*—To test the spreading power 0.5 cc. of the azoserum preparation was mixed in a syringe with 0.25 cc. of India ink previously diluted with 2 volumes of water, and the mixture inoculated intracutaneously into the dorsal part of the flank of a rabbit.<sup>4</sup> The injection of this amount of azoserum was

<sup>4</sup> The direction and ultimately the extent of the spread is influenced by gravity.

followed by a slow and progressive spread of the substance (as shown by inoculation made without indicator) and of the ink particles as well. In the rabbit the maximum area of diffusion is reached ordinarily after 5 to 6 hours, but diffusion may progress for even longer periods. The type of progression of the spreading has been illustrated in Text-fig. 1, and in Figs. 1 and 2.



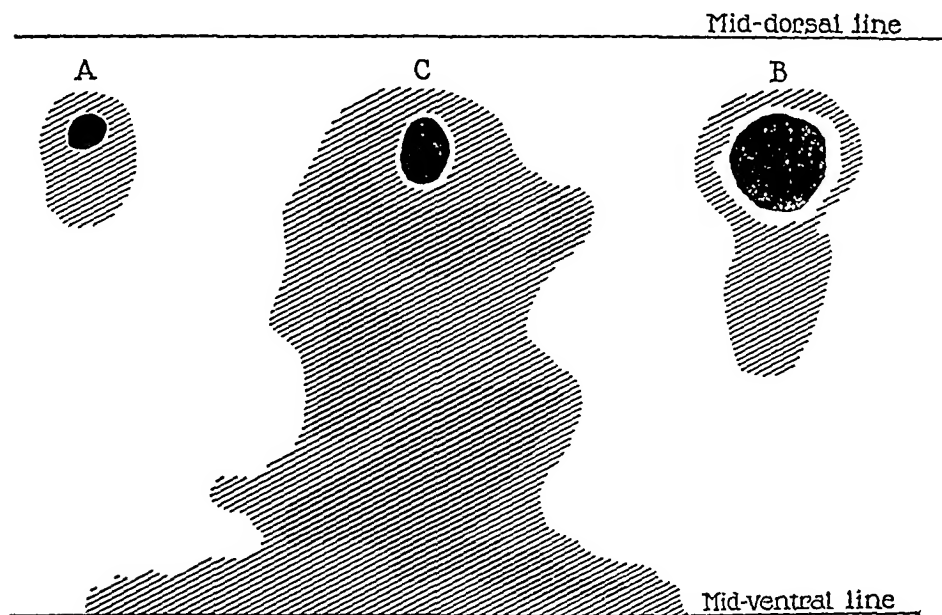
TEXT-FIG. 1. Progression of spreading of India ink particles in the rabbit skin under the influence of azoprotein (sulfanilic acid egg albumin).

A = saline control. B = spreading of 0.5 cc. azoalbumin plus 0.25 cc. of India ink indicator. 1 = site of inoculation. 2, 3, 4, and 5 = measurements after 2, 4, 7, and 9 hours following inoculation, respectively.

The diffusion extended over an area of 82 to 133 sq. cm., while the controls made with Ringer's solution or untreated horse serum reached a spread of 6 to 10 sq. cm. The extent of maximum diffusion may vary greatly from one individual to another owing to differences in the original permeability of the skin used for the test.

The types of spreading induced by sulfanilic acid and its diazo and azo derivatives are compared in Text-fig. 2 and in Table I.

The condition resulting from the inoculation of sulfanilic acid alone did not differ from that produced by saline solutions or normal horse serum. Uncoupled aromatic compounds and nitrites were eliminated as a cause of spreading when it was found that when phenylalanine, which cannot be diazotized, was submitted to the usual manipulations



TEXT-FIG. 2. Character of spreading induced by sulfanilic acid derivatives. Intradermal inoculation in the rabbit of 0.5 cc. of solution plus 0.25 cc. of India ink indicator.

A = 1.5 per cent sulfanilic acid solution (8.2 sq. cm.); B = 1.5 per cent diazo compound solution (24.2 sq. cm.); C = azoserum preparation (126.7 sq. cm.).

of diazotization and coupling, the resulting mixture of amino acid and nitrites caused a spread of India ink no greater than that induced by isotonic saline. Uncoupled proteins were eliminated as a cause of spreading when horse serum or protein solutions gave the same results as isotonic saline. As mentioned above, the diazo derivative produced a massive spread of the ink particles immediately around the point of injection while further diffusion seemed to be prevented.

On the other hand, azoprotein solutions are apt to spread slowly

through the skin, carrying with them the ink particles, which accumulate in places quite remote from the site of inoculation. Often, as in the case of Text-fig. 2 and of Fig. 3, the diffusion seemed to be limited ultimately by anatomical conditions of the body and the position of the animal.

In contrast with the rubefacient effect of the uncoupled diazo compound, azosera were not injurious to the skin, except for an edematous

TABLE I

*Area of Spread in the Rabbit Skin Resulting from the Intradermal Inoculation of 0.5 Cc. of Sulfanilic Acid Derivatives plus 0.25 Cc. of India Ink Indicator*

| Experiment No. | Ringer's solution | Normal horse serum | Sulfanilic acid (neutral solution) 1.5% | Diazo compound (neutral solution) 1.5% | Sulfanilic acid azosera |
|----------------|-------------------|--------------------|---|--|-------------------------|
|                | sq. cm.           | sq. cm.            | sq. cm.                                 | sq. cm.                                | sq. cm.                 |
| 1              | 6.1               |                    | 6.5                                     | 17.5                                   | 67.6                    |
| 2              | 9.3               |                    | 10.0                                    | 23.0                                   | 84.0                    |
| 3              | 8.3               |                    | 6.4                                     | 22.5                                   | 117.6                   |
| 4              | 7.4               |                    | 6.0                                     | 16.2                                   | 123.0                   |
| 5              | 5.2               |                    |   | 20.1                                   | 90.8                    |
| 6              | 10.0              |                    | 10.8                                    | 24.5                                   | 131.1                   |
| 7              | 1.8               |                    |   |  | 90.3                    |
| 8              | 6.0               |                    |   |  | 90.1                    |
| 9              | 5.5               |                    |   |  | 87.0                    |
| 10             |                   | 7.5                | 7.0                                     | 25.0                                   | 133.6                   |
| 11             |                   | 8.1                | 10.8                                    | 26.3                                   | 96.0                    |
| 12             |                   | 7.5                | 7.0                                     | 18.8                                   | 105.0                   |
| 13             |                   | 7.8                | 6.6                                     | 37.8                                   | 97.0                    |
| 14             |                   | 6.7                |   | 19.2                                   | 109.0                   |
| 15             |                   | 8.4                |   | 23.2                                   | 82.0                    |
| 16             |                   | 2.0                |   | 16.2                                   | 105.3                   |
| Average....    | 6.6               | 6.8                | 6.8                                     | 22.3                                   | 100.6                   |

thickening persisting only during active spreading. The spreading power of the azosera solution was retained after complete dialysis. Neutral solutions of azosera stored in the ice box retain their spreading power up to 10 months after preparation, whereas drying, completed at 105°C., destroys about half the spreading activity.

*Effect of Azosera on the Growth of Bacteria in Vitro.*—When neutral azosera solutions of graded potency were mixed with equal volumes of bacterial suspensions, subcultures to agar and to broth always grew



as abundantly as subcultures from bacterial suspensions mixed with equal volumes of isotonic saline. For these tests 24 hour agar cultures were taken up in 100 cc. distilled water or in isotonic saline and after different periods of contact with azoserum, from 2 minutes to 2 hours, at room temperature, the mixtures were subcultured at 37.5°C. for 24 hours. In five experiments the growth of these cultures was consistently abundant. The results are shown in Table II.

As shown in Table II, neutral azoserum solutions have no inhibiting effect on the growth *in vitro* of *Staphylococcus aureus* and *albus*, *B. coli*, or *B. prodigiosus*. The cultures obtained from azoserum mixtures were always as abundant as those obtained from the saline controls.

*Azoalbumin*.—Azoalbumin was prepared according to the procedure adopted for azoserum: 10 cc. of a 3 per cent albumin solution (soluble albumin egg, Merck) was treated with a quantity of diazo compound equivalent to about 2.0 millimoles. The amount of alkali required for the coupling of pure protein solutions was generally less than when serum was used, possibly because of the presence of additional buffer agents in the serum.

The diffusion obtained with this material was of the same type, both in character and in extent, as that induced by the usual azoserum preparations. 0.5 cc. of this azoalbumin solution gave a spread of 126 sq. cm. upon inoculation in the rabbit skin. Azoalbumin solutions stored in the ice box retained their diffusing power. They exhibited no rubefacient effect on the skin and were without any bactericidal power *in vitro*, as tested for the effect on the growth of *Staphylococcus aureus* cultures.

The correlation between the potency of azoprotein solutions and the amount of diazo compound used in their preparation was also investigated. When the concentration of albumin was kept constant and the concentrations of diazonium salt used for coupling were graded up to a quantity equivalent to 2 millimoles of diazo compound for 10 cc. of 3 per cent egg albumin the spreading power of the coupled products was found to be proportional to the quantity of diazo compound used for coupling.

According to Pauly (8) the chemical groups in proteins most apt to react with diazo compounds are tyrosine and histidine. Moreover, Pauly showed that each molecule of these amino acids can unite with

TABLE II  
*Properties of Diazo and Azo Compounds and Their Effect on the Growth of Bacteria in Vitro*

| Solutions tested     | Concentration of chemical in solution | Growth of bacteria in agar and broth |                             |                |                       | Oxidation of KI-starch reagent | Reaction with "R" salt* | Spreading area |
|----------------------|---------------------------------------|--------------------------------------|-----------------------------|----------------|-----------------------|--------------------------------|-------------------------|----------------|
|                      |                                       | <i>Staphylococcus aureus</i>         | <i>Staphylococcus albus</i> | <i>B. coli</i> | <i>B. prodigiosus</i> |                                |                         |                |
|                      | per cent                              |                                      |                             |                |                       |                                |                         | sq. cm.        |
| Saline.....          | 1.0                                   | +++++                                | +++++                       | +++++          | +++++                 | —                              | —                       | 9.2            |
| Sulfanilic acid..... | 1.0                                   | —                                    | —                           | —              | —                     | —                              | —                       | 8.5            |
| Diazo compound.....  | Half coupling                         | +++++                                | +++++                       | +++++          | +++++                 | ++                             | ++                      | 22.7           |
| Azoserum I.....      | Full coupling†                        | +++++                                | +++++                       | +++++          | +++++                 | ++                             | ++                      | 43.5           |
| Azoserum II.....     | 1.5 coupling                          | +++++                                | +++++                       | +++++          | +++++                 | —                              | —                       | 66.8           |
| Azoserum III.....    | Double coupling                       | +++++                                | +++++                       | +++++          | +++++                 | —                              | —                       | 82.0           |
| Azoserum IV.....     |                                       | +++++                                | +++++                       | +++++          | +++++                 | —                              | —                       | 105.3          |

\* The "R" salt (2-naphthol 3-6-sodium disulfonate) gives a dark colored product with diazo compounds (18). This reaction should be positive when an excess of diazonium salt has been used for coupling.

† Full coupling refers to a proportion of about 1.2 millimoles of diazo compound to 10 cc. of horse serum, when the action with "R" salt was moderately positive. In the case of double coupling, 10 cc. of serum were treated with twice that amount of diazo compound.

two molecules of the free diazo group. However, aliquot molecular concentrations of diazonium salt and of these amino acids in the isolated state yielded no more than 20 per cent of the quantity of azo compound expected from theoretical consideration (9). This discrepancy was attributed to concomitant side reactions.

The amount of both tyrosine and histidine available in 10 cc. of 3 per cent egg albumin (*i.e.* 0.3 gm. albumin) would correspond approximately to a total of 0.1 millimole for the two amino acids (10). From these considerations, complete diazotization would be provided with 1.0 millimole of the diazo compound. For the tests recorded in Table III, 3 per cent protein solutions were used. In each case, 1.0 millimole of the diazonium salt was made to react with 10 cc. of the protein solution. The tests were performed as usual by intradermal inoculation of 0.5 cc. of the solution in the rabbit skin. In this case, the area of spread from azoalbumin solution was 76.7 sq. cm. However, more active preparations result when even higher concentration of the diazo reagent is provided, the maximum effect being obtained when there is a proportion of 2 millimoles of the diazonium salt per 0.3 gm. of egg albumin. Such preparations gave a spread of from 126 to 137 sq. cm. in the rabbit skin. This seems to be the saturation point for the protein since even greater excess of diazide fails to increase the potency of the preparation but leaves a toxic solution, indicating the presence of free diazo groups.<sup>5</sup>

These observations may suggest that, under the present conditions, the yield in complete diazotization of albumin, is no more than 10 per cent, or that there are in the protein molecule groups other than tyrosine and histidine able to unite with the diazo radical.

*Azogelatin.*—Gelatin is known to lack certain amino acids: tyrosine is absent, but as much as 2.9 per cent of histidine has been reported (10, 11). However, the ability to unite with diazo compounds proved to be limited, at least with the grade of gelatin used in these experiments, the histidine content being apt to vary from one brand of gelatin to another. When a 3 per cent gelatin solution was used instead of horse serum or albumin, a moderately colored product was

<sup>5</sup> Decomposition of the diazo compound is indicated by abundant effervescence occurring during the process of coupling with proteins.

obtained and the azogelatin, after dialysis, gave an average spread in the rabbit skin of 31.4 sq. cm.

Azogelatin prepared according to the foregoing method exhibited a definite oxidizing power, indicating the presence of free diazo compounds in the solution. These preparations prevented the growth of staphylococcus *in vitro*, neutralized vaccine virus suspensions, and had a rubefacient effect on the skin. Non-toxic azogelatin preparations could probably be prepared if care were taken to avoid an excess of reagent and if the concentration of diazo compound were limited to the coupling power of the gelatin molecule.

TABLE III

*Properties of Azo Compounds Prepared by Coupling Diazobenzenesulfonic Acid with Various Proteins*

| Type of protein       | Oxidation of KI-starch reagent | Inhibition of growth of <i>Staphylococcus aureus</i> <i>in vitro</i> | Area of intradermal spread in rabbit skin<br><i>sq. cm.</i> | Skin reaction                |
|-----------------------|--------------------------------|--|---|------------------------------|
| Gelatin.....          | +++                            | +++  | 31.7  | Redness, edematous in center |
| Casein.....           | —                              | —  | 68.4  | Redness, edematous in center |
| Egg albumin I*.....   | —                              | —  | 76.7  | Normal, no irritation        |
| Egg albumin II*.....  | —                              | —  | 137.0   | Normal, no irritation        |
| Horse serum.....      | —                              | —  | 67.3  | Normal, no irritation        |
| Chicken serum.....    | —                              | —  | 74.2  | Normal, no irritation        |
| Saline (control)..... | —                              | —  | 8.1   | —                            |

\* 1.0 and 2.0 millimoles of diazonium salt to 10 cc. of 3 per cent egg albumin solution were used in the preparation of egg albumin I and egg albumin II respectively.

The spreading power of azogelatin solutions could not be increased by the use of stronger alkali or by treating the protein with larger amounts of diazo compound. The foregoing observations sustain the opinion that the spreading power of azoprotein depends on the number of diazo groups attached to the protein molecule.

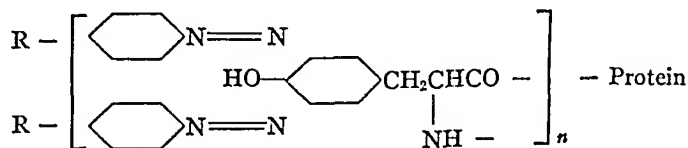
*Specificity of the Diazonium Radical on the Spreading Power of Azoprotein*

Azoproteins were found to possess the property of increasing dermal permeability irrespective of the type of protein to which the diazo

compound was attached. The rôle played by the azo group in the spreading property of azoproteins was indicated when it was found that the side chains in the aromatic nucleus of the diazonium radical did not specifically affect the spreading power of the azoprotein.

Equimolecular amounts of aniline and of arsanilic acid were diazotized in the usual manner. The diazo compounds were then coupled in various proportions either with horse serum or with egg albumin or casein. The resulting azoproteins, as well as the intermediate products of diazotization, were tested as usual for their ability to diffuse through the rabbit skin. The results, presented in Table IV, demonstrate that the effects obtained with sulfanilic acid derivatives can be duplicated with aniline and arsanilic acid derivatives. When the proportion of reagents was comparable, *i.e.* 2.5 millimoles of the diazo compound for each 10 cc. of serum, the spreading power of the product appeared to be of the same type and intensity (sera III, Table IV) whether the azoprotein was derived from aniline, arsanilic acid, or sulfanilic acid. It may be mentioned that some disadvantage attends the relatively low solubility of the aniline azoserum. The fact that the structure of the diazonium radical is not directly involved in the diffusing property of azoproteins is further illustrated by the behavior of some other azoproteins prepared from benzene polypeptide derivatives, whose effects are also presented in Table IV.

Therefore it seems that the property conferred upon azoprotein by coupling results from the introduction of the azo group in the molecule. According to the foregoing observations, active azoproteins may be presented by the following formula, where histidine (or possibly some other cyclic grouping) could be substituted for tyrosine and where R could be a group such as sulfonic acid, arsonic acid—or hydrogen in the case of aniline.



Moreover, the spreading power of the azoprotein appears to increase with the number of azo groups entering the molecule. Assuming that the conditions are uniform throughout the solution, it may be that only

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one active hydrogen of the reacting protein amino acid will be first substituted if an insufficient amount of diazo reagent be provided. The maximum effect would be reached with the saturation of the coupling power of the protein.<sup>6</sup>

TABLE IV\*  
*Spreading Power of Diazo and Azo Compounds Other than Sulfanilic Acid Derivatives*

| Solution tested for spreading power†                  | Concentration in solution | Diazo compound per 10 cc. serum | Area of spread |
|---|---------------------------|---------------------------------|----------------|
|   | per cent                  | millimoles                      | sq. cm.        |
| Aniline derivatives:                                  |                           |                                 |                |
| Ringer's solution (control).....                      | 1.0                       |                                 | 6.3            |
| Diazobenzene hydroxide.....                           |                           |                                 | 35.7           |
| Azoprotein (horse serum) I.....                       |                           | 0.6                             | 39.0           |
| Azoprotein (horse serum) II.....                      |                           | 1.2                             | 47.0           |
| Azoprotein (horse serum) III.....                     |                           | 2.5                             | 90.0           |
| Arsanilic acid derivatives:                           |                           |                                 |                |
| Ringer's solution (control).....                      | 1.7                       |                                 | 4.6            |
| Arsanilic acid (neutral solution).....                | 1.7                       |                                 | 6.0            |
| Diazo compound.....                                   |                           | 0.6                             | 27.0           |
| Azoprotein (horse serum) I.....                       |                           | 1.2                             | 55.0           |
| Azoprotein (horse serum) II.....                      |                           | 2.5                             | 73.8           |
| Azoprotein (horse serum) III.....                     |                           |                                 | 109.6          |
| Azoprotein (casein).....                              | 4.0                       |                                 | 62.6           |
| Amino acid derivatives (azo compounds):‡              |                           |                                 |                |
| Aminobenzoyl-glutaminic acid-horse serum.....         | 4.0                       | 1.2                             | 30.1           |
| p-aminobenzoyl-leucyl-glycyl-leucine-horse serum..... | 4.0                       | 0.6                             | 24.0           |
| p-aminobenzoyl-diglycyl-glycine-horse serum.....      | 3.8                       | 0.6                             | 29.5           |

\* The tests were made as usual by inoculating intradermally in the rabbit 0.5 cc. of the test solution together with 0.25 cc. of the India ink indicator. Measurements were made the following day.

† The azoprotein preparations were obtained by coupling with 10 cc. horse serum or a 3 per cent protein solution.

‡ I am indebted to Drs. K. Landsteiner and J. van der Scheer who kindly provided the samples for these tests.

<sup>6</sup> In their study on the antigenic power of azoproteins, Landsteiner and Lampl (12) found that intensive treatment of the protein with diazo compounds did not seem to favor the production of antibodies, better results being obtained when a lesser proportion of the diazo compound to protein had been used for the preparation of the antigen. This discrepancy might suggest that antigenic property goes with partial coupling of the protein amino acids while the spreading power is directly proportional to the degree of azotization of the protein.

## DISCUSSION

Diazonium derivatives of aromatic amines were discovered by Griess (13), and the ability of these compounds to form highly colored products under certain conditions has been utilized extensively in the dye industry. Ehrlich showed that this property of yielding colored compounds could be applied to the diagnosis of certain pathological conditions (14). More recently this chemical reaction supplied valuable material for the study of immune processes (15-17).

In the work here reported it was found that azoproteins injected intracutaneously were capable of spreading through an extensive area of the skin. The protein acquires this property upon coupling with an aromatic compound through a diazo group, but the entering aromatic radical fails to confer any specific character on the spreading phenomenon. Likewise, the type of protein selected for the reaction is indifferent provided the nature of the protein itself does not exclude the formation of azo combinations. The presence of the azo linkage in the protein molecule appears to be the factor responsible for the appearance of spreading properties and, within certain limits, the spreading power depends upon the number of diazo groups introduced into the molecule. The effect of coupling on the physical properties of the protein and its relation to the diffusing capacity of the product has not been investigated. The introduction of a strong acid radical such as sulfonic acid or arsonic acid is likely to affect the solubility and also the ionization of the protein. However, this does not seem to explain entirely the phenomenon, since the azoproteins prepared from aniline exhibit comparable diffusing properties. The direct proportionality between the concentration of dialyzed azoproteins and their power to diffuse might indicate that we are dealing here with a physical rather than a chemical phenomenon.<sup>7</sup>

The mechanism of spread of azoproteins has not been closely investigated. Microscopic examination of the treated area shows that dispersion occurs within the connective tissue layer, the epithelial components of the skin remaining unaffected. During active spread the collagen bundles appear swollen and more widely separated from each other.

<sup>7</sup> Indirect observations suggest that the osmotic pressure of azoproteins is appreciably greater than that of the original proteins.

So far there is no evidence that azoproteins and testicle extract have a common mechanism of action, although their ultimate effect on tissue permeability may be the same.

In the beginning of this work, some spreading was observed following the injection of diazo derivatives. It is reasonable to assume that when the diazonium salt was introduced into the neutral or slightly alkaline medium of the skin some coupling took place at the expense of the body proteins. The diffusion observed would then be produced by the newly formed azoproteins rather than by the free diazide.

#### SUMMARY

1. Azoproteins are shown to have the property of spreading when introduced intradermally into the rabbit skin.

2. The aromatic derivative selected for the coupling does not affect specifically the spreading property of the azoprotein. Likewise, the type of protein has no importance, except in quantitative respects.

3. The spreading property conferred upon a protein by coupling appears to derive from the presence of the azo group.

4. The spreading power of an azoprotein preparation seems to be determined by the number of diazo groups which enter the protein molecule, and to vary in direct proportion with the concentration of the solution.

5. Azo compounds of low molecular weight fail to exhibit any significant effect on skin permeability.

It is a pleasure to thank Dr. D. A. MacFadyen for his assistance and advice in the preparation of the manuscript.

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#### EXPLANATION OF PLATE 13

FIG. 1. Rabbit 1 (left side). Spread produced by intracutaneous inoculations of India ink suspension plus: *A*, 0.5 cc. Ringer's solution; *B*, 0.5 cc. azoserum I (prepared by coupling 0.6 millimole diazobenzenesulfonic acid with 10 cc. horse serum proteins); *C*, 0.5 cc. azoserum II (1.2 millimoles diazo reagent coupled with 10 cc. horse serum proteins). Photographs were taken 2 hours after injection. Final measurements of the area of spread, made the next day, were 6.7, 45.5, and 18.2 sq. cm. respectively.

FIG. 2. Rabbit 1 (right side). Spread of India ink indicator produced by intradermal inoculation of: *A*, 0.5 cc. Ringer's solution; *B*, 0.5 cc. azoserum III (1.8 millimoles diazo compound coupled with 10 cc. horse serum proteins); *C*, 2.5 millimoles diazo compound coupled with 10 cc. horse serum proteins. Photographs taken 3 hours after inoculation. Final measurements made the next morning were 6.0, 82.6, and 94.9 sq. cm. respectively.

FIG. 3. Rabbit 2. Spread produced by intracutaneous inoculation of 0.25 cc. of India ink indicator plus: *A*, 0.5 cc. normal horse serum; *B*, 0.5 cc. azoserum (2.5 millimoles diazobenzenesulfonic acid coupled with 10 cc. horse serum proteins); *C*, 0.5 cc. azo-egg albumin (from coupling of 2.5 millimoles diazobenzene-sulfonic acid with 10 cc. 3 per cent egg albumin solution). The photographs were taken 24 hours after injection. Under the influence of the azoproteins the indicator spread extensively through the skin, in the main part following the direction of gravity, and the India ink particles were found accumulated in the lowest part of the abdomen.



Photographed by Joseph B. Haulenbeck

(Claude: Spreading property of azoproteins)



# ISOLATION OF POLIOMYELITIS VIRUS FROM THE NASOPHARYNX\*

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The number of times that the virus of poliomyelitis has been isolated from the nasopharynx of individuals either ill or in contact with this disease is small.<sup>1</sup> It is so small, in fact, that it is questionable whether the epidemiology of poliomyelitis will be readily elucidated through this approach unless it is found that the infrequent detection of the virus in the nasopharynx is the result of inadequate methods. In an effort to improve these methods, therefore, the investigations described in this paper have been carried out, the opportunity having been presented by the epidemic of poliomyelitis which occurred in California during the summer of 1934. As a result of this study another case of poliomyelitis which harbored the virus in the throat was found. The circumstances under which the virus was isolated will be described.

## *Methods*

*Clinical Material.*—Nasopharyngeal washings were obtained from patients in various stages of suspected or diagnosed cases of poliomyelitis. A few determina-

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\* The participation of Dr. George Parrish and the Los Angeles City Department of Health in furnishing laboratory space, animal quarters, personnel, transportation, and enthusiastic cooperation is gratefully acknowledged. The courtesies of the Los Angeles County General Hospital and staff in permitting clinical observation of cases and study of material is likewise appreciated.

<sup>1</sup> The virus of poliomyelitis has been isolated, according to our estimation, thirteen times from the throat or tonsils of living human beings, and eleven (or perhaps thirteen) times from the nasopharynx or tonsils of fatal human cases. Eight positive results have been obtained from patients in, or prior to the acute stage of the disease (2-5); two from convalescents, one early (17 days) (6) and the other late (5 months) (7); and three from what appear to be healthy contacts (8-10).

tions were also made on contacts. Choosing and classifying cases for study proved difficult owing to the fact that the great majority of diagnosed cases in the epidemic were mild. According to Kessel, Hoyt, and Fisk (1), the mortality rates and per cent of cases with residual paralysis or pleocytosis of spinal fluid were exceptionally low, while the number of ailments which were diagnosed poliomyelitis among adults and hospital attendants was extraordinarily high.

Our cases have been classified on the basis of the following diagnostic terminology: (1) paralytic, *i.e.* cases with paralysis developing in association with the usual signs of the disease, and positive spinal fluid findings, such as a pleocytosis (more than 12 cells) or an increase of globulin; (2) abortive, *i.e.* cases in which paralysis did not develop but which presented positive spinal fluid findings in association with characteristic symptoms and signs; (3) suspected abortive, *i.e.* cases with characteristic symptoms or signs similar to those in the abortive group in which lumbar punctures were either not done, or in which the results were negative.

*Preparation of the Inoculum.*—Sterile physiological saline solution was used as the irrigating medium in the majority of instances. The washings were prepared for inoculation by (a) phenolization, (b) filtration, and (c) glycerinization.

(a) *Phenolization.*—Samples of freshly obtained washings generally amounting to from 10 to 20 cc. were employed. Within 1 to 2 hours of the time of their collection 5 per cent phenol was added in an amount sufficient to make a final concentration of 0.5 per cent. The material was then allowed to stand at room temperature for at least 30 minutes before being inoculated into the monkey.

(b) *Filtration.*—Samples of freshly obtained nasopharyngeal washings generally amounting to about 10 to 15 cc. were centrifuged at about 1,600 R.P.M. for 10 to 20 minutes. The supernatant fluid, still turbid, was filtered under the following conditions: Seitz filters with disc 2.3 cm. in diameter and a chamber of 15 cc. capacity, measuring 1.5 x 10.5 cm., were used. 10 to 15 cc. of beef infusion broth, pH 7.8, was first passed through the filter at 15 pounds pressure. This procedure required not more than 5 minutes. The test material was then filtered at 30 pounds pressure. The lapse of time for this process varied from 5 to not more than 30 minutes. The filtrate was immediately used for inoculating purposes.

(c) *Glycerinization.*—Samples of nasopharyngeal washings, generally amounting to about 10 to 15 cc., were centrifuged as stated above. The supernatant fluid was removed and usually set aside for filtration (method given above). The sediment consisting of small flakes of mucopurulent exudate, cells, and bacteria, was transferred with a capillary pipette either immediately to 2 or 3 cc. of a solution of 50 per cent glycerine in saline, or it was left in the ice box for from 1 to 4 days and was subsequently transferred to the glycerine solution.

Before inoculating the sediment it was washed once in 10 to 15 cc. of saline solution. The washed sediment, in a volume from 0.2 to 0.5 cc., was inoculated intracerebrally, and the remaining supernatant saline solution (9 to 14 cc.) was usually

inoculated intraperitoneally. All material eventually tested by this method had remained in glycerine for more than 12 days.

*Inoculations.*—Small *rhesus* monkeys weighing on an average from 4 to 5 pounds were used. Inoculations with phenolized and filtered material were performed within 3 to 4 hours after collection and were made under ether anesthesia by the intracerebral (0.2 to 1 cc.) and intraperitoneal routes (2 to 30 cc.). Inoculated monkeys were observed daily, and daily temperature records were taken over a period of from 3 to 4 weeks. Twenty animals which failed to show symptoms after 3 weeks were used again. Furthermore, in ten instances the same animal was inoculated at from 2 to 5 day intervals with similarly treated material from two or three different patients who represented similar types of cases. Thus, one monkey would receive, at 2 or 3 day intervals, inoculations of filtered material from contacts; another, inoculations of phenolized material from suspected abortive cases.

Single inoculations were employed with phenolized and filtered material. When glycerine had been used as a preservative for the inoculum, the initial inoculation was often reinforced by a second intracerebral and intraperitoneal inoculation given 7 to 8 days later.

If any of the inoculated animals died within 3 weeks of the last inoculation from some cause other than poliomyelitis, the experiment was considered unsatisfactory and was discarded. All animals which showed symptoms of poliomyelitis, or remotely suggesting poliomyelitis, were sacrificed at what appeared to be an appropriate time, and material from the mid-brain, medulla, and cervical, thoracic, and lumbar regions of the spinal cord was preserved in 50 per cent glycerine, 10 per cent formalin, and 95 per cent alcohol. Histological sections stained with hematoxylin and eosin were made from the material preserved in formalin, and in those in which lesions of poliomyelitis or lesions resembling poliomyelitis were found, sections stained in toluidine blue were prepared from the material in alcohol. Criteria for the establishment of the diagnosis of poliomyelitis employed in these experiments were that: (1) after an incubation period of from 4 to 12 days the animal showed acute fever with subsequent defervescence, tremor, ataxia, or paralysis (they were sacrificed shortly afterwards inasmuch as none of the animals died which contracted the experimental disease on the first passage); (2) lesions typical of poliomyelitis were found in the cord; and (3) the strain was brought at least through its second passage. Criteria for a negative result were that the animal did not die from some cause other than poliomyelitis during a period of 3 weeks from the last inoculation, and that it failed to show the first two criteria requisite for a positive result.

*Passage Experiments.*—Samples of medulla and upper cervical cord, usually weighing from 0.7 to 1.5 gm., were ground in a mortar with sand or alundum in saline solution to make a 10 per cent emulsion. 1 cc. was inoculated intracerebrally and the remainder of the emulsion inoculated intraperitoneally. If the animal failed to show symptoms within 6 days, a second inoculation (reinforce-

ment) was made on the 6th, 7th, or 8th day from a freshly prepared suspension of medulla and cord.<sup>2</sup> Animals thus inoculated were observed and sacrificed in the same manner as those which received human material.

### RESULTS

These may be summarized by the brief statement that the virus of poliomyelitis was isolated from the nasopharynx of a single individual with an illness which conformed in its symptomatology to a case of suspected abortive poliomyelitis. It was not detected in the small number of frank (abortive) cases tested, or in healthy contacts. The type of clinical material studied and the methods employed in each appear in Table I. Here the cases have been listed according to the day of illness on which washings were obtained. Experiments which were considered unsatisfactory because of the premature death of the monkey have not been included in this list.

*Description of Case Which Yielded the Virus.*—The virus was obtained from the nasopharynx on the 1st day of a brief illness. Details of this illness can perhaps be best presented in terms of the family history (Text-fig. 1).

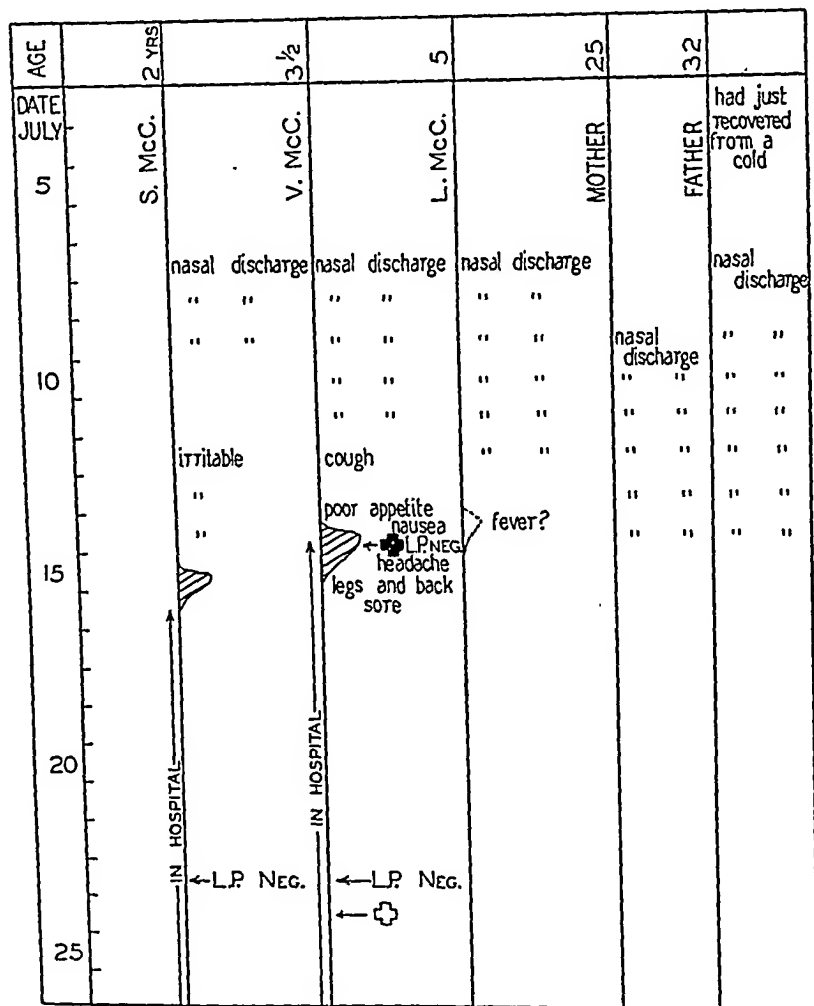
*Family History.*—V. McC., aged 3½ years, was one of three children. The family resided in Los Angeles when poliomyelitis became epidemic in that city; *i.e.*, during the late spring and summer months of 1934. Its members had all been well during May and June with the exception of the father who had contracted a "cold" in the last week of June. Between July 7 and 9 all of the family members, including the father, suffered from "colds" characterized chiefly by nasal discharge. This symptom lasted from 3 to 7 days. On July 14, V. developed fever, headache, anorexia, nausea, and pains in the legs and back. She was admitted to the Los Angeles County General Hospital on the same day, with the diagnosis of suspected poliomyelitis. On admission the child presented the usual signs of an acute, mild febrile (temperature 101.4°) illness. The tonsils were large and red and in addition her neck was slightly stiff. However, her spine was flexible and there were no abnormal reflexes or muscle weaknesses. Nasopharyngeal washings taken at this time contained an excess of mucopurulent material. A lumbar puncture performed at this time, and also a second one performed 9 days later, were negative.

On July 15 the child's younger brother S., aged 2 years, also developed fever

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<sup>2</sup> It has been our experience that in early (second through fourth) passages of strains isolated from human cases, if a single inoculation of a 10 to 20 per cent cord emulsion is used, positive results have occurred in but 45 per cent of the animals inoculated. If double inoculations of a similar dose are employed, positive results have occurred in 90 per cent.

and symptoms similar to those of his sister. He was admitted to the hospital on July 16. A lumbar puncture, performed on July 23, was negative. In both children the illness did not last more than 48 hours.



⊕ VIRUS PRESENT IN WASHINGS

L.P. = LUMBAR PUNCTURE

⊖ VIRUS NOT DETECTED IN WASHINGS

TEXT-FIG. 1. Schematic diagram of illnesses which occurred in the McC. family. The vertical lines represent individual members of the family; their respective ages appear at the top. The shaded areas indicate roughly the time of onset and duration of fever in the two youngest children.



A second nasopharyngeal washing was obtained from V. on the 10th day after the onset.

*Experimental Details.*—The volume of the saline washings obtained on July 14 from V. McC. totalled 18 cc. They were divided into two parts, A and B. To part A (6 cc.) 0.5 per cent phenol was added and (after standing for 45 minutes) 1 cc. was inoculated intracerebrally and 5 cc. intraperitoneally into one monkey (No. C-2-9). The remaining 12 cc. of the washings (part B) was centrifuged, the supernatant fluid was removed for filtration, and the sediment designated as part C. The supernatant fluid (part B) was filtered through a Seitz filter (see under Methods) and of this filtrate 1 cc. was immediately inoculated intracerebrally and 7 cc. intraperitoneally into one monkey (No. C-3-1). Both of these animals (Nos. C-2-9 and C-3-1) had been inoculated within a week previously with material which was subsequently shown not to contain the virus. Part C, the sediment from 12 cc. of the original washings in saline, was placed in the ice box and after 3 days was transferred to 50 per cent glycerine. On July 27 (13 days after its collection), 0.5 cc. of the washed sediment was inoculated into each of two monkeys (Nos. C-4 and C-1-4). All of the animals (Nos. C-2-9, C-3-1, C-4, and C-1-4) developed experimental poliomyelitis.

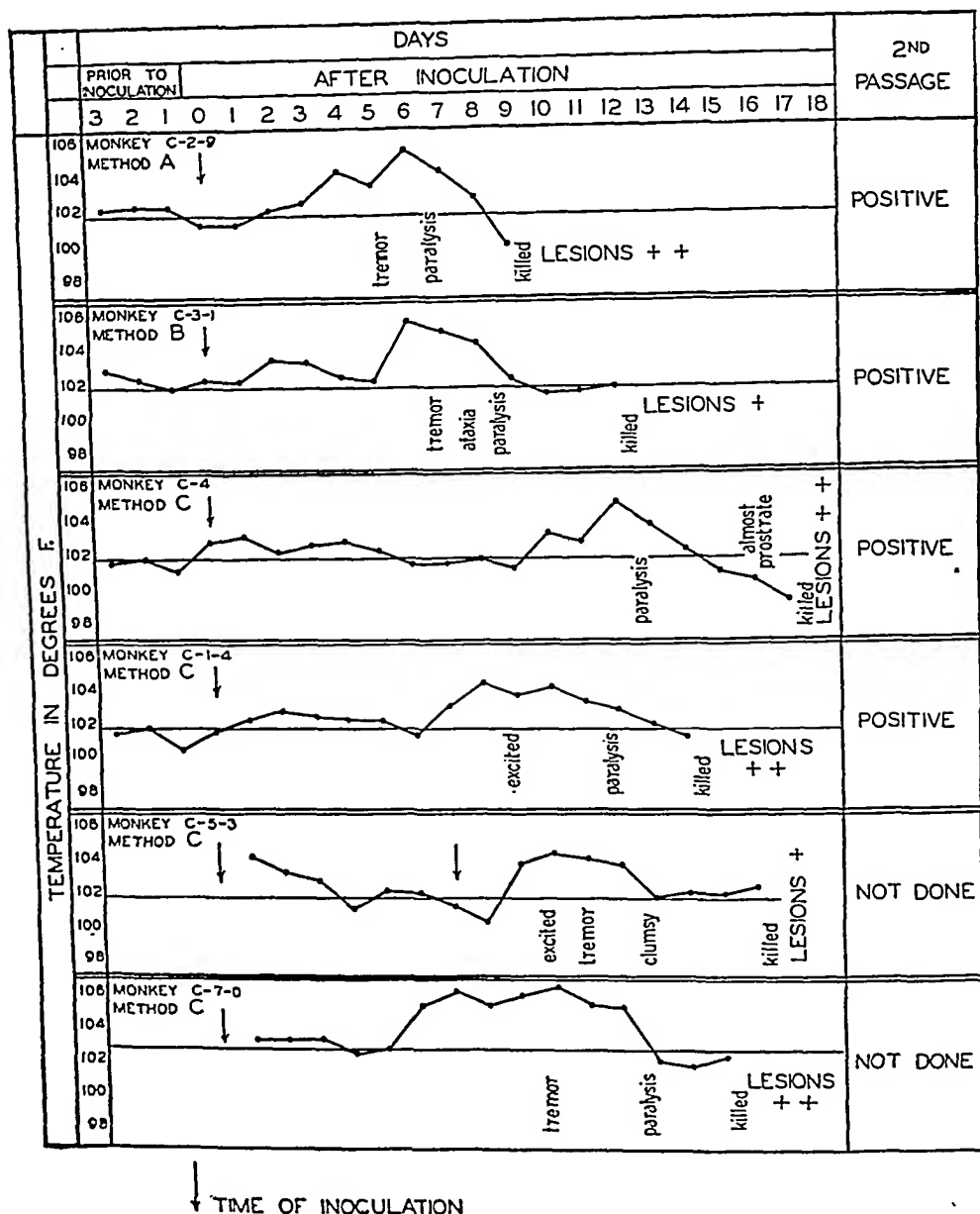
Subsequently (after 39 days), 0.3 cc. of the washed sediment from glycerine was inoculated intracerebrally and 12.0 cc. of the supernatant intraperitoneally into one monkey (No. C-5-3) with subsequent reinforcement, after a weekly interval. Again after 101 days a single inoculation was made in another monkey (No. C-7-0). Both of these animals developed experimental poliomyelitis.

Samples of the washings taken on the 11th day of the illness (July 24) were divided into two parts, A and B. A was treated with 0.5 per cent phenol and inoculated into one monkey; B was filtered and inoculated into one monkey. The former animal died on the 3rd day and the latter failed to develop the experimental disease.

*Evidences of the Experimental Disease in Monkeys Inoculated with Material from Case of V. McC.*—Details of the course of the experimental disease exhibited by the six monkeys inoculated with material from the first washings from V. McC. appear in Text-fig. 2. The incubation period to the onset of fever ranged from 4 to 10 days. All of the animals developed either tremor, ataxia, or paralysis in varying degree. All probably would have recovered had they not been sacrificed as soon as signs of recovery were apparent. In all of these six animals characteristic lesions were present in the spinal cord and medulla. The strains from four of the animals (Nos. C-2-9, C-3-1, C-4, and C-1-4) were brought through the second passage. Two of these strains were subsequently brought to the fourth and seventh passages.

A small quantity of the original glycerinized sediment from the nasopharyngeal washings from this case, and 10 per cent emulsions of spinal cord representing early passages of this strain of virus were instilled into the nose of three monkeys. The results were negative.

Small quantities of the original glycerinized sediment were also inoculated



TEXT-FIG. 2. Temperature charts from six monkeys inoculated with material from the first washings from V. McC., all of which developed experimental poliomyelitis.

TABLE I

## Clinical and Experimental Data on Nasopharyngeal Washings

| Case | Initials | Age<br>yrs. | Type of polio-<br>myelitis or clinical<br>diagnosis | Day<br>of<br>ill-<br>ness | Evi-<br>dences of<br>upper<br>respira-<br>tory<br>inflam-<br>mation<br>(a) | Patient's<br>temper-<br>ature<br>when<br>irrigated | Lumbar puncture<br>when irrigated<br>(b) | Irrigating<br>fluid | Method<br>(c)                           | Amount inoculated                                   |  | Monkey<br>No.                                    | Results in<br>monkey              |                 |                |
|------|----------|-------------|---|---------------------------|--|--|--|---------------------|---|---|--|--|-----------------------------------|-----------------|----------------|
|      |          |             |   |                           |  |  |  |                     |   | Intracere-<br>brally                                | Intraperitoneally                                  |  | Fever<br>(d)                      | Symptoms<br>(e) | Lesions<br>(f) |
| 1    | V. McC.  | 3½          | Suspected<br>abortive                               | 1                         | +  | 101.4  | Negative                                 | Saline              | A<br>B<br>C-13<br>C-13<br>C-39<br>C-101 | cc.<br>1.0<br>1.0<br>0.5<br>0.5<br>0.3 + 0.2<br>0.4 | cc.<br>5.0<br>7.0<br>0<br>0<br>12.0 + 11.0<br>10.0 | C-2-9<br>C-3-1<br>C-4<br>C-1-4<br>C-5-3<br>C-7-0 | +4<br>+6<br>+10<br>+7<br>+9<br>+6 | +               | +              |
| 2    | E. R.    | 9           | "   | 1                         | +  | 103.4  | "  | "                   | A<br>C-121                              | 0.8<br>0.4 + 0.4                                    | 10.0<br>10.0 + 10.0                                | C-1-9<br>C-7-2                                   | -<br>-                            | -               | 0<br>0†        |
| 3    | J. Hn.   | 2           | "   | 1                         | +  | 103.4  | Not done                                 | "                   | A<br>B<br>C-30                          | 1.0<br>1.0<br>0.4 + 0.3                             | 6.0<br>5.0<br>12.0 + 11.0                          | C-4-6<br>C-4-5<br>C-5-6                          | -<br>±7<br>±6                     | -               | 0<br>-<br>0†   |
| 4    | J. S.    | 3           | Abortive  | 1                         | +  | 101.0  | 38 W. B. C.<br>Glob. +                   | Water               | A<br>B<br>C-76                          | 1.0<br>1.0<br>0.2 + 0.4                             | 13.0<br>10.0<br>9.0 + 9.0                          | C-3-8<br>C-3-7<br>C-5-4                          | -<br>-<br>-                       | -               | -<br>0<br>0    |
| 5    | A. G.    | 10          | Suspected<br>abortive<br>(familial ex-<br>posure)   | 1                         | -  | 103.0  | Not done                                 | Saline              | B<br>C-75                               | 1.0<br>0.3 + 0.4                                    | 7.0<br>9.0 + 9.0                                   | C-3-9<br>C-6-4                                   | -<br>-                            | -               | 0<br>0         |
| 6    | P. G.    | 7           | Abortive  | 1                         | ?  | 100.0  | 39 W. B. C.<br>Glob. +                   | Broth               | B<br>C-70                               | 1.0<br>0.4 + 0.4                                    | 2.0<br>9.0 + 9.0                                   | C-3-7<br>C-6-3                                   | -<br>-                            | -               | 0<br>0         |

Tested individually

| 7  | Z. D.  | 15 | Abortive   | 1 | +  | 102.4 | 450 W. B. C.<br>Glob. + | Saline | B<br>C-66 | 1.0<br>0.3 + 0.5 | 2.0<br>9.0 + 9.0   | C-3-7<br>C-6-2 | 0<br>0  |
|----|--------|----|--|---|----|-------|-------------------------|--------|-----------|------------------|--------------------|----------------|---------|
| 8  | J. C.  | 11 | Suspected<br>abortive<br>(acute ton-<br>sillitis?) | 1 | ++ | 103.0 | Not done                | "      | C-130     | 0.4 + 0.4        | 10.0 + 10.0        | C-7-3          | 0†      |
| 9  | J. Hy. | 1  | Suspected<br>abortive                              | 2 | ?  | 99.2  | "                       | "      | B<br>C-32 | 1.0<br>0.5 + 0.3 | 4.0<br>12.0 + 11.0 | C-4-2<br>C-5-4 | +8<br>— |
| 10 | G. S.  | 1½ | "  | 2 | ?  | 102.0 | Negative                | Water  | B         | 1.0              | 3.0                | C-4-0          | —       |
| 11 | D. B.  | 7  | "  | 2 | ++ | 99.4  | Not done                | "      | C-140     | 0.3 + 0.3        | 10.0 + 10.0        | C-7-7          | —       |

\* (c) Symptomatology of monkey:

- + Paralyzes or ataxia.
- ± Nervous, agitated.
- No symptoms suggestive of experimental poliomyelitis.

\* (f) Lesions in the central nervous system:

- ++ Extensive lesions in medulla and spinal cord.
- + Few lesions.
- ± Very scanty lesions.
- No lesions.
- 0 Animal not sacrificed.
- 0† Monkey subsequently infected with a strain of poliomyelitis virus isolated from the 1934 California epidemic.

\* (c) Method of preparing inoculum:

- A, 0.5 per cent phenol added.
- B, Seitz filtration (concentration *in vacuo* in a few of the pooled samples).
- C-15, sediment preserved in 50 per cent glycerine,—inoculated 15 days after collection of washings.

D, untreated washings.

\* (d) Type of fever in monkey:

- +5 Fever lasting more than 2 days with onset 5 days after inoculation.
- ± Fever lasting 1 or 2 days.
- No fever.

\* (a) Clinical appearance of throat:

- ++ Red throat, exudate present.
- + Red throat.
- ± Questionable red throat.
- Normal throat.
- ? Throat not seen.

\* (b) Lumbar punctures were considered negative when: (1) the cell count was below 12 white blood cells per c. mm. and (2) the Pandy test was negative.

TABLE I—*Concluded*

| Case      | Initials | Age<br>yrs. | Type of poliomyelitis or clinical diagnosis | Day of illness | Evidences of upper respiratory inflammation* <sup>(a)</sup> | Patient's temperature when irrigated | Lumbar puncture when irrigated* <sup>(b)</sup> | Irrigating fluid | Method* <sup>(c)</sup> | Amount inoculated |                   | Monkey No. | Results in monkey     |                          |                         |
|-----------|----------|-------------|---|----------------|---|--------------------------------------|--|------------------|------------------------|-------------------|-------------------|------------|-----------------------|--------------------------|-------------------------|
|           |          |             |   |                |   |                                      |  |                  |                        | Intracerebrally   | Intraperitoneally |            | Fever* <sup>(d)</sup> | Symptoms* <sup>(e)</sup> | Lesions* <sup>(f)</sup> |
| 12        | P. C.    | 4           | Abortive                                    | 2              | +   | °F. 102.0                            | 109 W. B. C. Glob. —                           | Water            | C-140                  | cc. 0.5 + 0.4     | cc. 10.0 + 10.0   | C-7-5      | —                     | —                        | 0                       |
| 13        | R. B.    | 9           | "   | 3              | +   | 100.0                                | 79 W. B. C. Glob. +                            | Broth            | B                      | 0.9               | 1.0               | C-7        | —                     | —                        | —                       |
| 14        | N. K.    | 19          | "   | 3              | +   | 101.6                                | 210 W. B. C. Glob. +                           | "                | C-96                   | 0.3 + 0.4         | 9.0 + 9.0         | C-6-5      | —                     | —                        | —                       |
| 15        | G. M.    | 17          | Suspected abortive                          | 4              | +   | 101.2                                | Negative                                       | Saline           | B                      | 1.4               | 2.4               | C-6        | —                     | —                        | —                       |
| 16        | L. N.    | 3           | Abortive                                    | 4              | ++  | 101.0                                | 38 W. B. C. Glob. +                            | Water            | C-140                  | 0.4 + 0.3         | 10.0 + 12.0       | C-6-4      | —                     | —                        | 0                       |
| 17        | D. R.    | 20          | Suspected abortive                          | 4              | ±   | 99.0                                 | Not done                                       | "                | C-147                  | 0.4 + 0.4         | 10.0 + 10.0       | C-7-3      | —                     | —                        | 0†                      |
| 18        | A. W.    | 5           | "   | 4              | +   | 103.6                                | "  | "                | C-140                  | 0.3 + 0.5         | 10.0 + 10.0       | C-7-6      | —                     | —                        | 0                       |
| 19        | E. M.    | 12          | "   | 5              | +   | 102.4                                | Negative                                       | Saline           | B                      | 0.9               |                   | C-5        | —                     | —                        | 0                       |
| 1 Re-peat | V. McC.  | 3½          | "   | 11             | —   | 98.4                                 | "  | "                | B                      | 1.0               | 3.0               | C-4-7      | —                     | —                        | 0                       |
|           | Jo. S.   | ½           | Contact                                     | ?              |   |                                      |  | "                | C-41                   | 0.4 + 0.3         | 12.0 + 11.0       | C-5-7      | —                     | —                        | —                       |

Tested individually—*Concluded*

|    |        |    |                             |   |   |       |                      |        |         |           |                         |       |     |
|----|--------|----|-----------------------------|---|---|-------|----------------------|--------|---------|-----------|-------------------------|-------|-----|
| 20 | J. B.  | 4} | Suspected abortive          | 2 | ± | 99.2  | Not done             | Saline | A       | 0.8       | 20.0                    | C-3-4 | -   |
| 21 | L. B.  | 5} | "                           | 1 | - | 101.8 | "                    | "      | B       | 0.8       | 10.0                    | C-3-3 | -   |
| 22 | R. S.  | 9  | Abortive                    | 3 | + | 101.0 | 49 W. B. C. Glob. +  | Broth  | C-42    | 0.5 + 0.3 | 12.0 + 11.0             | C-5-5 | -   |
| 14 | N. K.  |    | As stated above—see Case 14 |   |   |       |                      |        |         |           |                         |       |     |
| 11 | D. B.  |    | As stated above—see Case 11 |   |   |       |                      |        |         |           |                         |       |     |
| 12 | P. C.  |    | "                           | " | " | "     | "                    | "      | B       | 1.0       | 3.0                     | C-9   | -   |
| 16 | L. N.  |    | "                           | " | " | "     | "                    | "      | D       | 0.5       | 5.0                     | C-8   | ±   |
| 17 | D. R.  |    | "                           | " | " | "     | "                    | "      | B and D | 0.8       | 10.0 (intra-tracheally) | C-1-0 | ±   |
| 18 | A. W.  |    | "                           | " | " | "     | "                    | "      | B       | 0.8       | 1.5                     | C-1-1 | -   |
| 23 | E. C.  |    |                             |   |   |       |                      |        | C-163   | 0.5 + 0.6 | 10.0 + 10.0             | C-6-9 | ±   |
| 24 | A. F.  | 36 | Suspected abortive          | 2 | ± | ?     | Negative             | Water  | B and D | 0.8       | 7.0 (intra-tracheally)  | C-1-8 | -   |
| 25 | R. So. | 21 | "                           | 8 | + | 97.6  | Not done             | "      | B       | 0.8       | 0.5                     | C-1-9 | -   |
| 26 | H. W.  | 13 | "                           | 2 | + | 101.5 | Negative             | "      |         |           |                         |       | -   |
| 27 | S. T.  | 24 | "                           | 8 | + | 100.0 | Not done             | "      |         |           |                         |       | -   |
|    |        | 19 | Abortive                    | 7 | + | 100.2 | 156 W. B. C. Glob. + | Saline | A       | 0.8       | 30.0                    | C-3-6 | -   |
|    |        |    |                             |   |   |       |                      | "      | B       | 1.0       | 11.0                    | C-3-5 | +15 |
|    |        |    |                             |   |   |       |                      |        | C-41    | 0.4 + 0.3 | 12.0 + 11.0             | C-5-0 | ±26 |
|    |        | 3} | Contact                     |   |   |       |                      | Broth  | B       | 1.0       | 13.0                    | C-2-2 | -   |
|    | L. F.  | 10 | "                           |   |   |       |                      |        |         |           |                         |       | -   |
|    | C. F.  |    | 5 suspected contacts        |   |   |       |                      | Broth  | B       | 1.0 + 1.0 | 6.0 + 8.0               | C-2-7 | +23 |
|    |        |    | 12 contacts                 |   |   |       |                      |        | B       | 1.0 + 1.0 | 3.0 + 4.0               | C-2-8 | -   |
|    |        |    | 3 normal                    |   |   |       |                      | Water  | B       | 1.0       | 5.0                     | C-1   | -   |

intracerebrally, intraperitoneally, and intranasally into many white mice. It was also inoculated into the cornea of the rabbit's eye. The results were uniformly negative.

*Questionable Results.*—A few results of this type occurred in these experiments and are listed under Results in monkey in Table I. None of them fulfilled all three criteria necessary to establish the diagnosis; namely: (1) that the animal should show signs compatible with the experimental disease, such as fever, tremor, ataxia, or paralysis; (2) that lesions of poliomyelitis should be demonstrated in the spinal cord; and (3) that the strain should be brought to the second passage. Of the several questionable results obtained, only one series of tests deserves attention, namely, that with pooled material from Cases 11, 12, 16, 17, and 18 (see Table I). Material (preserved in glycerine for 140 to 147 days) from each of these individual cases was subsequently tested, with negative results. This experiment has not been considered significant except that it illustrates some of the difficulties and pitfalls in this type of investigation.

#### DISCUSSION

The experience described in this report confirms the view that the virus of poliomyelitis cannot readily be obtained from the nasal passages of living persons. It cannot yet be decided, however, whether the difficulty is due to absence of virus or to unsatisfactory technique. The facilities for this investigation were excellent but the epidemic was mild. A search for the poliomyelitis virus in the living still needs to be made during a severe epidemic, by means of an experimental method the sensitivity of which can be determined.

#### SUMMARY

A single example of mild illness diagnosed as suspected abortive poliomyelitis is described in which the virus of poliomyelitis was recovered from the nasopharynx by three different methods. Failure to recover virus from a total of twenty-six cases diagnosed as suspected or abortive poliomyelitis and fourteen contacts is also reported.

The original material from the nasopharynx of the positive case proved unusually infective for the monkey, apparently even more so than are the majority of suspensions of spinal cords from fatal human cases of poliomyelitis. An explanation of this fact is not clear.

The method of isolating human virus from the throat, by means of preserving the sediment of washings from this site in glycerine, has been shown to be efficient in one case for a period of 101 days.

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# STUDIES ON THE MECHANISM OF PRODUCTION OF A SPECIFIC BACTERIAL ENZYME WHICH DECOMPOSES THE CAPSULAR POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS

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In previous papers (1, 2) methods have been described for the preparation of a bacterial enzyme capable of decomposing the capsular polysaccharide of Type III Pneumococcus. Although the micro-organism from which this enzyme is extracted grows readily and abundantly on common bacteriological media, the production and recovery of satisfactory yields of active enzyme are conditioned by at least two sets of factors which affect the metabolism of the bacterial cells.

1. The specific enzyme is formed in appreciable amounts only when cultural conditions are such that the bacillus is compelled to utilize the Type III polysaccharide as its main source of energy for growth.

2. For a given amount of Type III polysaccharide decomposed by the bacillus, the yield of specific enzyme *recovered* from the culture increases directly with the rate of decomposition of the substrate in the course of growth.

Of the cultural conditions studied, two were found to increase greatly the rate of decomposition of the polysaccharide, and therefore the yield of enzyme: (a) incubation of the culture in very shallow layers to provide aerobic conditions; (b) addition of small amounts of a yeast extract preparation to a synthetic medium consisting of mineral salts and the specific capsular polysaccharide.

The new method of production of the enzyme to be described in the following pages is based on the above mentioned observation; *i.e.*, the more rapidly the polysaccharide is decomposed in the culture

medium, the larger the yield of specific enzyme recovered from the microorganism. It is therefore important to emphasize again certain theoretical considerations which appear to account for this fact.

The bacterial cells producing the specific enzyme are known to undergo autolysis very rapidly in all media and especially in the synthetic culture medium. In fact, such cultures exhibit cell disruption and spore formation within 12 hours after inoculation. Furthermore, cell disruption is accompanied by the release of free enzyme in the medium, so that very soon this free enzyme competes with the microorganism for the specific substrate. Some of the polysaccharide is therefore decomposed by the free enzyme without the bacillus benefiting thereby, leaving less substrate available for the production of new enzyme. Also, the free enzyme expends itself, as it were, on the polysaccharide of the medium, and becomes inactivated in the course of incubation.

Other things being equal one might suppose that a maximum yield of enzyme would be recovered if complete decomposition of the polysaccharide could be achieved by the microorganism before any autolysis occurs, and therefore before any free enzyme is released in the medium.

Attempts to modify the culture medium brought about some increase in the yield of enzyme, but the results were not striking enough to warrant detailed presentation. The limiting factor in all cultural methods is the fact, stated above, that autolysis begins within 12 hours; that is, before all the capsular polysaccharide could possibly be decomposed by the living cells. It was then found that very rapid decomposition could be obtained by resuspending in a solution of capsular polysaccharide enormous numbers of young cells recovered from a peptone medium in which no enzyme is formed. The development and results of a method based on this observation are now to be described.

## EXPERIMENTAL

### *Methods*

In the present investigation the cultures used, the constituents of the media, the serological tests employed to follow the disappearance of the capsular polysaccharide, and the method of titration of enzy-

matic activity, were in all respects identical with those described in previous papers (1, 2).

Large numbers of young cells of the specific microorganism, the "S III bacillus," were obtained by growth in a solution of casein hydrolysate in tap water (1 or 2 per cent as stated in the description of experiment) adjusted at pH 7.0. To provide for aerobic conditions, the cultures are incubated in very shallow layers. After 16 hours incubation (using a very small inoculum) the bacilli are separated by centrifugalization and resuspended in sterile tap water. Cells obtained by this technique appear as long thin bacilli, without any evidence of spore formation. The collection of enormous numbers of bacilli involves the most exacting aseptic technique, and requires that the purity of the culture be checked at every step.

*Effect of the Size of the Inoculum on the Rate of Decomposition of the Capsular Polysaccharide by the S III Bacillus*

*Experiment 1.*—A medium of the following composition was prepared.

0.2 per cent  $\text{Na}_2\text{HPO}_4$   
0.1 per cent  $(\text{NH}_4)_2\text{SO}_4$   
0.005 per cent yeast extract  
0.1 per cent capsular polysaccharide  
Tap water

This medium was distributed in lots of 10 cc. each in 14 Erlenmeyer flasks (of 300 cc. capacity). These flasks were inoculated with varying numbers of bacterial cells recovered from a culture in casein hydrolysate medium. The amounts of cells used are presented in Table I, expressed in terms of original volume of casein hydrolysate culture.

The cultures in the 14 Erlenmeyer flasks were incubated at 37°C. and tested at intervals of time for the disappearance of the capsular polysaccharide. After 3 days incubation, the cultures were filtered through Berkefeld (V) filters and the filtrates titrated for enzymatic activity. (It has been shown previously that the enzyme passes readily through these filters.)

The time necessary for disappearance of the capsular polysaccharide from the medium, and the enzymatic activity of the filtrates expressed in minimal amounts required for the decomposition of 0.01 mg. of the polysaccharide in the presence of toluol, are presented in Table I.

The results of Experiment 1 demonstrate that when the number of cells used as inoculum is increased to a definite maximum, there is a progressive increase in both the rate of decomposition and the enzymatic activity of the culture filtrates. As will be noted, the smallest inocula used correspond to those employed in ordinary bacteriological

technique as, for instance, in the experiments described in previous papers (1, 2). For a given amount of capsular polysaccharide decomposed in the medium, the yield of enzyme obtained with small

TABLE I

*The Effect of Size of Inoculum on the Rate of Disappearance of the Polysaccharide from the Medium and on the Yield of Enzyme*

| Volume of specific synthetic medium | Inoculum expressed in terms of cc. of culture in casein hydrolysate | Time required for disappearance of polysaccharide in specific medium | Minimal amount of culture filtrate required to decompose 0.01 mg. polysaccharide |
|-------------------------------------|---|--|--|
| cc.                                 | cc.   | hrs.   | cc.  |
| 10                                  | 1   | 48   | 0.025  |
| 10                                  | 1   | 48   | 0.02   |
| 10                                  | 10  | 24   | 0.02   |
| 10                                  | 10  | 24   | 0.02   |
| 10                                  | 20  | 24   | 0.012  |
| 10                                  | 20  | 24   | 0.009  |
| 10                                  | 40  | 24   | 0.008  |
| 10                                  | 40  | 24   | 0.009  |
| 10                                  | 100   | Less than 12   | 0.005  |
| 10                                  | 100   | " " 12   | 0.003  |
| 10                                  | 200   | 8  | 0.002  |
| 10                                  | 200   | 8  | 0.002  |
| 10                                  | 400   | 8  | 0.002  |
| 10                                  | 400   | 8  | 0.002  |

inocula is only 10 per cent of the maximum yield obtained with the large inocula.

*Effect of the Presence or Absence of the Capsular Polysaccharide in the Medium on the Production of the Specific Enzyme*

It is apparent that enormous numbers of cells were used in the preceding experiment, especially since the bacillus grows very abundantly in the casein broth. It was therefore necessary to check once more whether or not the large yields of enzyme obtained were due simply to the presence of active enzyme in the cells used as inoculum.

*Experiment 2.*—In each of four Erlenmeyer flasks (300 cc. capacity) were placed 10 cc. of the following medium.

0.2 per cent  $\text{Na}_2\text{HPO}_4$   
 0.1 per cent  $(\text{NH}_4)_2\text{SO}_4$   
 0.005 per cent yeast extract  
 Tap water

To two of these flasks was also added the capsular polysaccharide in amounts sufficient to give a final concentration of 0.1 per cent.

Each of the four flasks received as inoculum the cells from 100 cc. of culture of the S III bacillus in 1 per cent casein hydrolysate broth. The cultures were incubated for 3 days at 37°C. and the enzymatic activity of the whole material (to eliminate the possibility of loss by filtration) was titrated in the presence of toluol to prevent further growth.

The results shown in Table II confirm the observation that the formation of appreciable amounts of active enzyme is conditioned by the presence of the specific substrate in the medium. It may be pointed out that the slight amount of additional growth afforded by the presence of 0.1 per cent polysaccharide in the medium is not sig-

TABLE II

*The Influence of the Presence or Absence of the Specific Polysaccharide in the Medium on the Yield of Enzyme*

| Medium   | Minimal amount of culture capable of decomposing 0.01 mg. polysaccharide in the presence of toluol |
|--|--|
|  | cc.  |
| Mineral medium.....                              | 0.2  |
| " " .....  | 0.4  |
| Mineral medium +0.1 per cent polysaccharide..... | 0.003  |
| " " +0.1 " " .....                               | 0.003  |

nificant when compared with the enormous number of cells in the inoculum.

Since the production of the specific enzyme is dependent upon the decomposition of the polysaccharide by the bacillus, it was of interest to test whether the yield of enzyme is a function of the concentration of polysaccharide in the medium.

*Experiment 3.*—10 cc. amounts of the medium described in Experiment 2 were distributed in Erlenmeyer flasks (300 cc. capacity); to these flasks was also added the capsular polysaccharide in varying amounts. Each flask was inoculated with the cells recovered from 200 cc. of a culture of the S III bacillus in 1 per cent casein hydrolysate broth. After 3 days incubation at 37°C. the cultures were filtered through a Berkefeld filter and the filtrates titrated for enzymatic activity (Table III).

It will be observed that as the concentration of specific substrate in the medium is increased, the yield of enzyme also increases until a

technique as, for instance, in the experiments described in previous papers (1, 2). For a given amount of capsular polysaccharide decomposed in the medium, the yield of enzyme obtained with small

TABLE I

*The Effect of Size of Inoculum on the Rate of Disappearance of the Polysaccharide from the Medium and on the Yield of Enzyme*

| Volume of specific synthetic medium | Inoculum expressed in terms of cc. of culture in casein hydrolysate | Time required for disappearance of polysaccharide in specific medium | Minimal amount of culture filtrate required to decompose 0.01 mg. polysaccharide |
|-------------------------------------|---|--|--|
| cc.                                 | cc.   | hrs.   | cc.  |
| 10                                  | 1   | 48   | 0.025  |
| 10                                  | 1   | 48   | 0.02   |
| 10                                  | 10  | 24   | 0.02   |
| 10                                  | 10  | 24   | 0.02   |
| 10                                  | 20  | 24   | 0.012  |
| 10                                  | 20  | 24   | 0.009  |
| 10                                  | 40  | 24   | 0.008  |
| 10                                  | 40  | 24   | 0.009  |
| 10                                  | 100   | Less than 12   | 0.005  |
| 10                                  | 100   | " " 12   | 0.003  |
| 10                                  | 200   | 8  | 0.002  |
| 10                                  | 200   | 8  | 0.002  |
| 10                                  | 400   | 8  | 0.002  |
| 10                                  | 400   | 8  | 0.002  |

inocula is only 10 per cent of the maximum yield obtained with the large inocula.

*Effect of the Presence or Absence of the Capsular Polysaccharide in the Medium on the Production of the Specific Enzyme*

It is apparent that enormous numbers of cells were used in the preceding experiment, especially since the bacillus grows very abundantly in the casein broth. It was therefore necessary to check once more whether or not the large yields of enzyme obtained were due simply to the presence of active enzyme in the cells used as inoculum.

*Experiment 2.*—In each of four Erlenmeyer flasks (300 cc. capacity) were placed 10 cc. of the following medium.

0.2 per cent  $\text{Na}_2\text{HPO}_4$   
 0.1 per cent  $(\text{NH}_4)_2\text{SO}_4$   
 0.005 per cent yeast extract  
 Tap water

To two of these flasks was also added the capsular polysaccharide in amounts sufficient to give a final concentration of 0.1 per cent.

Each of the four flasks received as inoculum the cells from 100 cc. of culture of the S III bacillus in 1 per cent casein hydrolysate broth. The cultures were incubated for 3 days at 37°C. and the enzymatic activity of the whole material (to eliminate the possibility of loss by filtration) was titrated in the presence of toluol to prevent further growth.

The results shown in Table II confirm the observation that the formation of appreciable amounts of active enzyme is conditioned by the presence of the specific substrate in the medium. It may be pointed out that the slight amount of additional growth afforded by the presence of 0.1 per cent polysaccharide in the medium is not sig-

TABLE II  
*The Influence of the Presence or Absence of the Specific Polysaccharide in the Medium on the Yield of Enzyme*

| Medium   | Minimal amount of culture capable of decomposing 0.01 mg. polysaccharide in the presence of toluol |
|--|--|
|  | cc.  |
| Mineral medium.....                              | 0.2  |
| " " .....  | 0.4  |
| Mineral medium +0.1 per cent polysaccharide..... | 0.003  |
| " " +0.1 " " " .....                             | 0.003  |

nificant when compared with the enormous number of cells in the inoculum.

Since the production of the specific enzyme is dependent upon the decomposition of the polysaccharide by the bacillus, it was of interest to test whether the yield of enzyme is a function of the concentration of polysaccharide in the medium.

*Experiment 3.*—10 cc. amounts of the medium described in Experiment 2 were distributed in Erlenmeyer flasks (300 cc. capacity); to these flasks was also added the capsular polysaccharide in varying amounts. Each flask was inoculated with the cells recovered from 200 cc. of a culture of the S III bacillus in 1 per cent casein hydrolysate broth. After 3 days incubation at 37°C. the cultures were filtered through a Berkefeld filter and the filtrates titrated for enzymatic activity (Table III).

It will be observed that as the concentration of specific substrate in the medium is increased, the yield of enzyme also increases until a



maximum is reached. Above a concentration of 0.1 per cent polysaccharide, the number of bacterial cells becomes the limiting factor, since experiments have shown that with concentrations of polysaccharide greater than 0.1 per cent larger numbers of cells are necessary to obtain maximum yields. However, under these conditions, the cell suspension becomes so heavy that it is no longer convenient for experimentation.

It may be mentioned at this point that any attempt to replace in the medium the capsular polysaccharide of Type III Pneumococcus by

TABLE III

*The Effect of the Concentration of Polysaccharide in the Medium on the Yield of Enzyme*

| Concentration of polysaccharide in the medium | Minimal amount of filtrate required for the decomposition of 0.01 mg. polysaccharide |
|---|--|
| <i>per cent</i>                               | <i>cc.</i>   |
| 0   | 0.3  |
| 0.01  | 0.03   |
| 0.04  | 0.008  |
| 0.1   | 0.002  |
| 0.2   | 0.002  |
| 0.4   | 0.005  |

related substances (polysaccharides from other types of Pneumococcus, gum arabic, glucuronic acid, glucose, etc.) was completely unsuccessful. This confirms once more the specificity of the phenomenon of enzyme production, as previously shown (1, 2).

*Effect of the Salt Concentration in the Medium on the Rate of Disappearance of the Capsular Polysaccharide and on the Yield of Enzyme*

It was observed that when very large inocula were used, as described in the preceding experiments, the presence or absence of yeast extract in the medium no longer affected the rate of decomposition of the polysaccharide or the yield of enzyme. When, however, the solution of mineral salts (0.2 per cent  $\text{Na}_2\text{HPO}_4$  + 0.1 per cent  $(\text{NH}_4)_2\text{SO}_4$  in tap water) was replaced by either distilled water or physiological saline, both the rate of decomposition and the yield of enzyme were unfavorably affected.

The following experiment was instituted to test whether the osmotic pressure of the medium influenced the activity of the cells.

*Experiment 4.*—To a 0.1 per cent solution of capsular polysaccharide in distilled water, varying amounts of sodium chloride, or a mixture of sodium phosphate and ammonium sulfate were added as described in Table IV. The media were distributed in 10 cc. amounts in Erlenmeyer flasks (300 cc. capacity) and inoculated with the cells recovered from 100 cc. of culture of the S III bacillus in casein hydrolysate broth. The rate of decomposition of the polysaccharide in the culture medium and the enzymatic activity of the culture filtrates are presented in Table IV.

TABLE IV  
*Influence of Salt Concentration of the Medium on the Metabolic Activity of the S III Bacillus*

| Salts   | Time required for disappearance of specific polysaccharide from the medium | Minimal amount of culture filtrate required for the decomposition of 0.01 mg. polysaccharide |
|---|--|--|
| <i>per cent</i>                                     | <i>hrs.</i>  | <i>cc.</i>   |
| 0.2 Na <sub>2</sub> HPO <sub>4</sub>                | 9  | 0.003  |
| 0.1 (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> |  |  |
| 1 NaCl  | 48   | 0.02   |
| 0.5 NaCl  | 24   | 0.1  |
| 0.3 NaCl  | 9  | 0.004  |
| 0.2 NaCl  | 9  | 0.002  |
| 0.1 NaCl  | 9  | 0.002  |
| 0   | 48   | 0.02   |

The results of Experiment 4 demonstrate that the activity of the S III bacillus in decomposing the capsular polysaccharide is greatest at an osmotic pressure corresponding to 0.1–0.2 per cent NaCl.

*Is the Production of the Specific Enzyme Dependent upon Cell Multiplication?*

From the results of the preceding experiments, it is apparent that when large numbers of cells recovered from a culture of the S III bacillus in a casein hydrolysate broth, and containing no appreciable amount of the specific enzyme, are resuspended in a solution of capsular polysaccharide containing 0.1 per cent NaCl, the specific substrate is rapidly decomposed and the cell suspension now exhibits marked enzymatic activity. To account for this observation, we may assume that the preexistent cells become so modified in the presence of the polysaccharide as to develop specific enzymatic activity; but it is also possible that the decomposition of the polysaccharide is brought about by a rapid growth of "new" cells which are the only

ones possessing enzymatic property. It is impossible to demonstrate the presence of newly formed cells by any method of numerical estimation, since the cells used as inoculum enormously outnumber those that might be present as a result of new growth. An attempt was made, therefore, to bring about the decomposition of the capsular polysaccharide by cells recovered from a culture in casein hydrolysate medium, under conditions such that cell multiplication could be excluded. For this purpose, mixtures of polysaccharide and bacterial cells were incubated: (a) at reactions outside the pH range suitable for the growth of the organism; (b) in the presence of antiseptics (phenol, toluol, chloroform, potassium cyanide); (c) under anaerobic conditions; (d) aerobically at 40°C. and at ice box temperature.

It must be stated here that the enzyme retains its specific activity under environmental conditions—the presence of antiseptics, anaerobiosis, a temperature of 40°C.—such as render the bacillus itself incapable of multiplying at the expense of the capsular polysaccharide. Under these conditions, in no instance did the polysaccharide disappear from the medium, and no enzymatic activity could be detected in the cultures. In other words, it was not possible to observe the formation of the specific enzyme in the absence of cell multiplication.

*Routine Method for the Production of the Specific Enzyme.*—As pointed out in Experiment 1, the decomposition of a definite amount of capsular polysaccharide by the use of very large numbers of bacterial cells under appropriate conditions yields an enzyme solution much more active than any obtained by ordinary cultural methods (2). The practical aspects of the new technique will now be briefly outlined.

The bacteria are grown in a solution of 2 per cent casein hydrolysate (pH 7.0) at 37°C. and under conditions of strict aerobiosis; the cells from the 16 hour old culture, separated by centrifugalization, are resuspended in small amounts of distilled water.

A medium is prepared consisting of 0.1 per cent capsular polysaccharide and 0.1 per cent NaCl in distilled water. This medium is distributed in 25 cc. amounts in large Erlenmeyer flasks (1 liter capacity) to provide for aerobic conditions, and each flask is inoculated with the cells recovered from 500 cc. of the culture of the S III bacillus in the casein hydrolysate medium. The material is incubated for 12–18 hours at 37°C. and the cultures tested to ascertain the disappearance of the specific polysaccharide and the absence of contaminants. The cultures are now frozen and thawed repeatedly to secure the release of the endocellular enzyme (2).

The enzyme is ultimately separated from the cell debris by filtration. However, since the cell suspension is very viscous, it is first subjected to the following treatment. The cell suspension is made alkaline to pH 10.0 by the addition of sodium borate. Equimolecular concentrations of dibasic sodium phosphate and calcium chloride are then added to bring about a heavy precipitate of calcium phosphate which facilitates the clarification of the material by centrifugalization; (it had been established previously that the enzyme is not adsorbed on the calcium phosphate at alkaline reaction). The supernatant which contains all the enzyme in solution is now passed through a Seitz filter,<sup>1</sup> then through a Berkefeld (V) filter. The potency of this filtrate is such that 0.002–0.004 cc. are required to decompose 0.01 mg. of the capsular polysaccharide under the conditions of the test. A method for the purification and concentration of this crude enzyme preparation will be described in the succeeding publication.

#### DISCUSSION

The microorganism which decomposes the capsular polysaccharide of Type III Pneumococcus was isolated by the use of a selective medium containing the specific polysaccharide as sole source of energy. This medium, although suitable because of its selectivity for the isolation of the pure culture, did not prove favorable for the growth of the organism and the production of the specific enzyme. The addition of small amounts of yeast extract served as an adjuvant to growth and brought about an increase both in the rate of decomposition of the polysaccharide in the medium, and in the yield of enzyme. For the reasons already stated this parallelism between the rate of decomposition and the yield of enzyme suggested that the maximum production of enzyme might be obtained if the cultural conditions were such as to bring about complete disappearance of the polysaccharide before autolysis had released any free enzyme in the medium. This result has been achieved by adding to a solution of the capsular polysaccharide at the proper osmotic concentration, young bacterial cells in numbers large enough to achieve decomposition in less than 12 hours.

The bacterial cells used for this purpose are obtained by growing the

<sup>1</sup> It is important to observe that the enzyme is completely adsorbed on the asbestos pad of the Seitz filter; this, however, can be prevented by washing the filter with nutrient meat infusion-peptone broth previous to filtration. After this treatment, the enzyme passes through the filter without loss.

S III bacillus in casein hydrolysate; in this medium the bacilli grow abundantly but do not form any appreciable amount of specific enzyme. The rapid development of the specific enzymatic activity in the presence of the polysaccharide, suggested that the cells recovered from the casein hydrolysate culture possessed a "latent" form of the enzyme which became "activated" in the presence of the specific substrate. Stephenson and Stickland (3) have already dealt with a similar phenomenon, the formation by *B. coli* of hydrogenlyase, an enzyme which liberates molecular hydrogen from formic acid. This enzyme is produced when the bacillus is grown in a medium containing sodium formate, but not in other media such as glucose or peptone broth. The authors concluded that the enzyme can be produced independently of cellular multiplication and that "enzyme formation is simply a chemical reaction between the medium and the cells."

Like hydrogenlyase, the specific enzyme produced by the S III bacillus belongs to the group of "adaptive" enzymes (4) which are formed only as a response to the specific substrate, but in the present instance it has not been possible to observe a production of active enzyme in the absence of cellular multiplication. It remains possible, however, that "activation" of the hypothetical "latent" form of the enzyme might be brought about by the use of a specific agent which would selectively inhibit multiplication without preventing the development of enzymatic activity.

#### SUMMARY

1. The microorganism which decomposes the capsular polysaccharide of Type III Pneumococcus grows abundantly in casein hydrolysate medium, but in this medium does not form any appreciable amount of the enzyme responsible for the decomposition of the polysaccharide.

2. When large numbers of cells grown in this medium are resuspended in a solution of the polysaccharide with the proper salt concentration, the specific substrate is rapidly decomposed, and filtered autolysates of the cell suspension exhibit marked enzymatic activity.

3. For a given amount of capsular polysaccharide decomposed in the medium, the yield of enzyme obtained by the present method is

## RENÉ DUBOS

much larger than that recovered by the cultural methods previously described.

4. Under the experimental conditions specified, the maximum yield of active enzyme is determined by two limiting factors; (a) concentration of the polysaccharide in the medium; (b) number of cells used as inoculum.

5. It was not found possible to induce the production of active enzyme in the absence of cellular multiplication.

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# THE USE OF GRADED COLLODION MEMBRANES FOR THE CONCENTRATION OF A BACTERIAL ENZYME CAPABLE OF DECOMPOSING THE CAPSULAR POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS

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The bacterial enzyme which decomposes the capsular polysaccharide of Type III Pneumococcus is an endocellular substance which is released from the specific bacterial cells by autolysis (1, 2) or by freezing and thawing (3).

The crude enzyme preparation is obtained as a cell-free filtrate which contains, in addition to the constituents of the medium, the metabolic products of the bacterial growth, and those products of autolysis which are capable of passing through Berkefeld and Seitz filters. The toxicity of this crude material has already been described (2), as well as earlier methods for its purification and concentration (2).

It has been found possible, for instance, to concentrate the enzyme to dryness, without appreciable loss of activity, by evaporation under reduced pressure and by precipitation in the cold with acetone, alcohol, and ammonium sulfate. As shown in a previous publication (2) it is also possible to concentrate the enzyme by ultrafiltration, using membranes of cellulose acetate which give an inactive ultrafiltrate because they hold back the active principle on their surface. This method combines in the same operation, concentration of activity, and some measure of purification by the elimination in the filtrate of much of the irrelevant material.

To give the best possible results, the method requires that membranes should be used with pores of the largest possible diameter compatible with recovery of the enzyme in the residue; this would increase the rate of filtration, and allow the removal of a greater



variety of extraneous substances, leaving the active principle itself in a purer state.

In recent years a method has been described by Elford (4) for the preparation of graded collodion membranes, the pores of which are remarkably uniform in size. The method has been used extensively for work with filterable viruses. However, since it is possible to prepare membranes covering a very wide range of pore size, the technique also permits of the separation of certain proteins in mixed solution. For instance ovalbumin can be separated from a mixture with hemoglobin by selective filtration through a membrane of proper pore diameter ( $7\text{ }\mu$ ). Under these conditions ovalbumin passes into the ultrafiltrate, whereas hemoglobin is held back on the membrane (5).

It has been observed that the bacterial enzyme which decomposes the capsular polysaccharide of Type III Pneumococcus is rapidly inactivated by crystalline trypsin and by commercial papain, at hydrogen ion concentrations at which the bacterial enzyme is very stable in the absence of the proteolytic enzymes.<sup>1</sup> This observation suggests that the activity is associated with a protein. An attempt was therefore made to subject the enzyme preparation to a process of selective ultrafiltration, in the hope that membranes would be found which would allow the passage of the autolytic products into the filtrate, but hold back on their surface the active enzyme-protein complex.

### *Methods*

*The Enzyme.*—The enzyme was prepared and titrated by the methods described in a previous paper (3). The method of preparation leaves the enzyme at an alkaline reaction which proves to be most favorable for filtration through the collodion membranes. The filtration experiments were carried out in the presence of chinosol, or preferably chloroform, antiseptics which do not exert any unfavorable action on the activity of the enzyme or on the properties of the membrane.

*The Filtration Equipment.*—The membranes were prepared and calibrated according to the description given by Bauer and Hughes (5); the filters used were those described by these same authors.

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<sup>1</sup> Unpublished observations. The preparation of crystalline trypsin was generously supplied by Dr. John Northrop, to whom the authors extend their heartiest thanks.

## EXPERIMENTAL

*Filtration End-Point.*—By filtration end-point is meant the smallest pore diameter which permits the test material to pass through the membrane and to appear in the filtrate.

*Experiment 1.*—In a first series of exploratory tests, the enzyme solution was filtered through a series of membranes with pore diameters ranging from 6.2  $\mu$  to 115  $\mu$ . Lots of 25 cc. of enzyme were subjected to ultrafiltration at 50 pounds pressure, and the filtration was interrupted after 20 cc. of filtrate had been collected in each case. Both the filtrates and residues were titrated for enzymatic activity. The average pore diameter of the membranes (A.P.D.) and the enzymatic activities

TABLE I  
*Ultrafiltration of the Bacterial Enzyme through Membranes of Different Pore Sizes*

| Membrane No.               | A.P.D. | Minimal amount required for the decomposition of<br>0.01 mg. capsular polysaccharide |                    |
|----------------------------|--------|--|--------------------|
|                            |        | Residue  | Filtrate           |
|                            | $\mu$  | $\mu$  | $\mu$              |
| 182                        | 115    | 0.0025*  | 0.0025             |
| 185                        | 72     | 0.005  | More than 0.1 cc.* |
| 150                        | 45     | 0.005  | " " 0.1 " *        |
| 121                        | 20     | 0.003  | " " 0.1 " *        |
| 211                        | 15     | 0.003  | " " 0.1 " *        |
| 210                        | 10.6   | 0.003  | " " 0.1 " *        |
| 125                        | 8.0    | 0.002  | " " 0.1 " *        |
| 143                        | 6.2    | 0.001  | " " 0.1 " *        |
| Control solution of enzyme |        |  | 0.002 cc.          |

\* This was the largest amount tested.

of the filtrates and residues, expressed in minimum amount required for the decomposition of 0.01 mg. of specific capsular polysaccharide, are presented in Table I.

The results of Experiment 1 show that the enzyme passed readily through pores of 115  $\mu$  diameter but was held back by membranes of smaller pore size. Contrary to expectations, however, titrations of activity carried out on the residue left on all the membranes of large pore diameter revealed that no concentration had taken place but rather a loss of enzyme. Membrane 143, with the smallest pore size (6.2  $\mu$ ), showed on the contrary a marked concentration of enzyme in the residue.

These observations suggested that adsorption of the enzyme had taken place in those membranes with pores of a diameter sufficient to allow the enzyme to penetrate, but not to pass through.

Elford (6) has recommended the use of meat infusion nutrient broth to reduce adsorption by the membrane during ultrafiltration. Although Bauer and Hughes (5) have observed that a preliminary passage of broth through the membranes alters the filtration end-point, it was decided to use this technique for studying the passage of the enzyme through membranes of different pore size.

TABLE II

*Filtration End-Point of the Bacterial Enzyme in the Presence of Broth*

| Membrane No.            | A.P.D. | Minimal amounts of the following materials required to decompose 0.01 mg. of polysaccharide |  |
|-------------------------|--------|---|--|
|                         |        | Filtrate  | Residue (resuspended in original volume of saline) |
|                         | $m\mu$ | cc.   | cc.  |
| 240                     | 28     | 0.002   | More than 0.1 cc.*                                 |
| 129                     | 24.4   | 0.002   | " " 0.1 " *  |
| 121                     | 20.4   | 0.002   | " " 0.1 " *  |
| 229                     | 17.6   | 0.002   | " " 0.1 " *  |
| 178                     | 14.8   | 0.002   | " " 0.1 " *  |
| 235                     | 12.8   | 0.002   | " " 0.1 " *  |
| 210                     | 10.6   | 0.005   | 0.005  |
| 232                     | 8.2    | More than 0.1 cc.*  | 0.002  |
| 143                     | 6.2    | " " 0.1 " *   | 0.002  |
| 144                     | 4.6    | " " 0.1 " *   | 0.002  |
| Control enzyme solution |        | 0.002   |  |

\* This was the largest amount tested.

*Experiment 2.*—50 cc. of nutrient meat infusion-peptone broth were added to 250 cc. of enzyme solution. Filters were fitted with membranes of pore diameters ranging from 4.6–28  $m\mu$ , and the whole apparatus carefully washed with broth. To each of these ultrafilters were added 25 cc. of broth-enzyme mixture and the filtration, carried out at 50 pounds pressure, was continued until all the fluid had been forced through the membranes. The membranes were then placed in 25 cc. saline (the original volume used) in an attempt to release any enzyme concentrated on the surface; both residues and filtrates were then titrated for activity (Table II).

This experiment confirms the finding that the presence of broth prevents or minimizes the adsorption of the enzyme on the collodion

membranes. With this technique, the enzyme passes completely through membranes with pores of  $12.8 \mu$  diameter or larger, and is completely held back by pores smaller than  $8.2 \mu$ . In the case of the latter membranes, the whole enzymatic activity can be recovered by merely redissolving the residue in saline or distilled water.

*Rate of Filtration of the Enzyme through a Collodion Membrane, and  
Example of Concentration*

In all the previous experiments, the filtration was limited to small amounts of enzyme solution; it was of interest to determine whether the accumulation of concentrated material on the surface of the membrane would inhibit further filtration.

TABLE III  
*Rate of Filtration of Large Volumes of Enzyme Solution through the Same Membrane  
(A.P.D.  $6.52 \mu$ ) Over a Period of 170 Hours*

| Time    | Total amount of filtrate | Rate per hr. |
|---------|--------------------------|--------------|
|         |                          | cc.          |
| hrs.    | cc.                      |              |
| 0-3     | 25.1                     | 8.4          |
| 3-6     | 39.8                     | 4.9          |
| 6-28    | 113.8                    | 4.7          |
| 28-52   | 210.8                    | 3.3          |
| 52-75   | 270.1                    | 2.6          |
| 75-97   | 328.8                    | 2.6          |
| 97-126  | 397.5                    | 2.3          |
| 126-170 | 491.5                    | 2.2          |

*Experiment 3.*—A filter was used of 100 cc. capacity, fitted with a membrane of 2.5 cm. diameter; the membrane (No. 119) had an average pore diameter of  $6.52 \mu$ . Filter and membrane were washed with nutrient broth and the filtration carried out at 50 pounds pressure. The filter was refilled several times and filtration continued for 170 hours. The rate of filtration is described in Table III.

The results presented in Table III show that, following a sharp initial drop in the rate of flow within the first 28 hours, the subsequent rate of filtration changes only slowly allowing the passage of fairly large volumes of fluid through the same membrane.

The pore size of the membrane was such that, according to previous results, the active enzyme could not pass into the filtrate. In an

attempt to recover the enzyme in solution, the membrane was washed three times with small amounts of distilled water, giving a final volume of 12 cc.; this corresponded to a 42-fold concentration (in volume) when compared with the original 491.5 cc. of enzyme solution.

The control enzyme solution, the ultrafiltrate, and the concentrate were titrated for enzymatic activity, and the results of the titration are given in Table IV.

Under the conditions of the experiment, it appears that ultrafiltration with a membrane of 6.2  $\mu$  average pore diameter gives a non-filterable residue, the activity of which is increased in direct proportion

TABLE IV

*Comparative Enzymatic Activity of the Ultrafiltrate and of a Solution of the Residue Representing a 42-Fold Concentration of Original Volume*

| Material                          | Minimal amount of material required for the decomposition of 0.01 mg. of capsular polysaccharide |
|-----------------------------------|--|
|                                   | cc.  |
| Original enzyme solution. . . . . | 0.005  |
| Ultrafiltrate. . . . .            | More than 1 cc.  |
| Concentrate. . . . .              | 0.00015  |

with the concentration in volume; the method is therefore available for the concentration of the crude enzyme preparations under practical conditions.

#### *Practical Aspects of the Method of Concentration by Ultrafiltration*

It has been shown that concentration of the enzyme has been obtained by the use of membranes of average pore diameter smaller than 8  $\mu$ ; in practice we have used membranes with pore diameters ranging from 5.5 to 7.5  $\mu$  without any loss of enzymatic activity.

For the concentration of amounts of enzyme larger than those required in the experimental work previously described, it was found necessary to increase the filtration area by using membranes of 6 cm. diameter. For that purpose, larger filters (750 cc. capacity), made of stainless steel, were used. Filtration was carried out at a pressure of 50 pounds obtained by the use of a reducing valve on the compressed air line. To minimize the possibility of contamination by air bacteria and particles of dust, the air was filtered through Seitz asbestos pads inserted between the air line and each individual filter. A number of technical difficulties were encountered, of which only two will be mentioned.

Although the body of the filter was made of stainless steel, the application of air pressure for filtration brought about an electrolytic corrosion at points of juncture of the different parts of the filters. This was eliminated by modifying the design so that the inside of the filter offered only a continuous, unbroken surface to the enzyme solution.

As stated before, chloroform is used as an antiseptic during filtration; it was therefore impossible to use as washers in the body of the filters any material such as rubber or leather containing substances soluble in chloroform. Washers made of heavy parchment paper proved very satisfactory as they are stable in the presence of chloroform and produce effective air-tight joints.

Under the conditions described, it took 5 days to filter to dryness 600 cc. of enzyme solution (previously saturated with chloroform). At the end of filtration, the membranes were washed several times in distilled water and all the activity thus recovered in solution. After filtration through Berkefeld V candles the enzyme preparation was frozen and in this state evaporated to dryness in vacuum (7, 8). This final product was redissolved and tested in animals to determine both its toxicity and activity *in vivo*. The results of these tests will be reported later.

#### DISCUSSION

The data presented in this paper indicate that, when proper precautions are taken to prevent adsorption of the active principle on the collodion membranes, the bacterial enzyme which decomposes the capsular polysaccharide of Type III *Pneumococcus* passes through pores of 10.6  $m\mu$  average diameter, but is held back by pores of 8.2  $m\mu$  diameter. When the enzyme thus held back on the membrane is placed again in the presence of water, it immediately goes into solution, indicating that the enzyme particles are not adsorbed in the pores, but are merely concentrated on the surface.

The sharpness of the filtration end-point, however, is not sufficient evidence for a determination of the actual size of the enzyme particle, since the shape of the particle and the play of electrical and other forces affect the possibility of its passage through pores of a definite diameter. It can only be stated that the property to decompose the polysaccharide of Type III *Pneumococcus* is carried by a protein molecule of not unusual dimensions.

In this respect it may be pointed out that Grabar and Riegert (9)

have subjected urease solutions to selective ultrafiltration through graded collodion membranes; they found that the enzyme is completely held back by membranes the pores of which are smaller than 30 m $\mu$ . Working with invertase prepared from the intestinal juice of the dog, Grabar (10) observed also that this enzyme is retained by pores of 10 m $\mu$  diameter while most of it passes through pores of 13 m $\mu$ .

Finally, it should be remarked that the membranes used for concentration have a pore diameter (5.5–7.5 m $\mu$ ) large enough to allow the passage of a certain number of proteins, such as egg albumin, for instance. It is therefore to be expected that the process of ultrafiltration separates from the active enzyme itself many of the split products of proteins and other autolytic and metabolic products of the cells. In a subsequent publication evidence of the purification will be shown in the decreased toxicity of the preparations thus obtained.

#### SUMMARY

1. The enzyme which decomposes the capsular polysaccharide of Type III Pneumococcus is associated with a protein which under optimal conditions of filtration passes through membranes with an average pore size of 10.6 m $\mu$  but is held back by pores having a diameter of 8.2 m $\mu$ .

2. When enzyme solutions are filtered to dryness through membranes of such porosity as to hold back the active principle, and when proper precautions are taken to prevent or minimize adsorption, the enzyme can be completely recovered in solution by immersing the membrane in distilled water or physiological salt solution.

3. These results are discussed with reference to the dimensions of the enzyme particle, and to the purification obtained in the course of ultrafiltration.

4. A practical method is described for the concentration and purification of the crude enzyme preparation by the use of graded collodion membranes.

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# SEROLOGICAL RELATIONSHIP BETWEEN PNEUMOCOCCUS TYPE I AND AN ENCAPSULATED STRAIN OF *ESCHERICHIA COLI*\*

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During the course of experimental work dealing with freshly isolated pneumococci, a culture was obtained which had been diagnosed serologically as Type I pneumococcus. Upon further examination the culture proved to be composed of Gram-negative bacilli, subsequently identified as *Escherichia coli*, which, however, were agglutinated by Type I antipneumococcic horse serum. The exact source of the organism has not been traced but presumably it grew out in a heart's blood culture from a mouse injected with sputum from a suspected case of lobar pneumonia.

The apparent serological relationship between this colon bacillus and Type I pneumococcus suggested the possibility of a situation somewhat analogous to that demonstrated by Avery, Heidelberger, and Goebel (1) between Type II pneumococcus and a member of the Friedländer group, later classified as Type B by Julianelle (2). This paper describes various characteristics of the colon bacillus, hereafter designated as Strain 8-a, and its connection with Type I pneumococcus.

*Morphological and Cultural Characteristics.*—The cultures used were transfers from a single, well isolated colony on an agar plate. Examination of Gram-stained smears suggested that the organism might be one of the colon-typhoid group. Eosin-methylene blue agar colonies were medium sized, flattened, and possessed the metallic sheen typical of colon bacilli. Acid and gas were formed in dextrose, maltose, mannite, and lactose broths, but not in sucrose. Brom-cresol purple milk was acidified and coagulated with gas bubbles in the curd.

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\* This is one of a series of studies on pneumonia being conducted under a grant from the Commonwealth Fund of New York.

Indol was formed, and nitrates were reduced to nitrites. Gelatin was not liquefied. Inulin was not attacked. There was no discoloration in lead acetate agar. In Russell's double sugar agar acid was formed throughout with gas bubbles in the butt of the tube. The organism was Voges-Proskauer negative and methyl red positive. Citrate was not utilized.

No motility was observed in preparations made on several different occasions.

Under the conditions usually employed it was not possible to demonstrate the presence of capsules. By the Neufeld method, however, using anti-*coli* 8-a rabbit serum, a definite *Quellung* reaction was obtained. For further evidence, 0.5 cc. of the culture was injected intraperitoneally into a mouse. 4 to 6 hours later the peritoneal exudate, when mixed with anti-*coli* 8-a rabbit serum and stained by Welch's method, showed the presence of distinct and well stained capsules surrounding the bacilli.

Well defined areas of hemolysis were formed around colonies in blood agar pour plates. Subsurface colonies had a gross appearance somewhat similar to those of pneumococci.

The characteristics described clearly permit the identification of this culture as an encapsulated strain of *Esch. coli* (*B. coli communis*).

*Virulence of Esch. coli* 8-a.—The intraperitoneal injection of 0.5 cc. of undiluted, and  $10^{-1}$  dilution of a  $4\frac{1}{2}$  hour blood broth culture killed duplicate white mice, weighing 16 to 20 gm., in less than 48 hours. Mice injected with higher dilutions survived for at least 72 hours. Plate counts indicated approximately 1 billion organisms per cc. in the original culture.

### *Agglutination Reactions*

Agglutination tests were conducted by mixing 0.5 cc. of serum dilutions with an equal amount of 18 hour broth cultures of the various organisms. The results recorded are readings made after 2 hours incubation in a 40°C. water bath. Storing the mixtures in the refrigerator overnight resulted in no essential changes in the relationships. Table I illustrates the cross-agglutination between Type I antipneumococcic horse serum and *Esch. coli* 8-a.

The results shown in Table I indicate that the non-specific agglutination in this instance is limited to Type I antipneumococcic horse serum since with Types II and III serums no reaction occurred with the colon bacillus and, curiously enough, Type I antipneumococcic rabbit serum also failed to agglutinate the organism. In order to determine whether the ability of Type I antipneumococcic horse serum to agglutinate *Esch. coli* 8-a is a general phenomenon tests were conducted with seven samples from different horse bleedings. Tests were also

made with two antimeningococcic serums, two samples of scarlet fever antitoxin, one of diphtheria antitoxin, and one of normal horse serum. All of the antipneumococcic horse serums agglutinated the colon bacillus to varying degrees, but none of the other serums used brought about the reaction. These results furnish added evidence that this

TABLE I

*Agglutination of Esch. coli 8-a in Type I Antipneumococcic Horse Serum*

| Antipneumococcic serums    | Cultures        | Final dilutions of serums |      |      |      |      |
|----------------------------|-----------------|---------------------------|------|------|------|------|
|                            |                 | 1:5                       | 1:10 | 1:20 | 1:40 | 1:80 |
| Pn I horse serum<br>D-30   | <i>Coli</i> 8-a | 4+                        | 4+   | 3+   | 2+   | 1+   |
|                            | Pn I            | 3+                        | 3+   | 3+   | 3+   | 2+   |
| Pn I horse serum<br>576-4  | <i>Coli</i> 8-a | 4+                        | 3+   | 3+   | 1+   | —    |
|                            | Pn I            | 2+                        | 3+   | 3+   | 3+   | 2+   |
| Pn I rabbit serum<br>R 241 | <i>Coli</i> 8-a | —                         | —    | —    | —    | —    |
|                            | Pn I            | 4+                        | 4+   | 4+   | 4+   | 3+   |
| Pn II horse serum<br>D-21  | <i>Coli</i> 8-a | —                         | —    | —    | —    | —    |
|                            | Pn II           | 4+                        | 4+   | 4+   | 1+   | —    |
| Pn II horse serum<br>546-2 | <i>Coli</i> 8-a | —                         | —    | —    | —    | —    |
|                            | Pn II           | 4+                        | 2+   | 1+   | —    | —    |
| Pn III horse serum<br>D-22 | <i>Coli</i> 8-a | —                         | —    | —    | —    | —    |
|                            | Pn III          | 4+                        | 3+   | 3+   | 2+   | 1+   |
| Pn III horse serum<br>D-39 | <i>Coli</i> 8-a | —                         | —    | —    | —    | —    |
|                            | Pn III          | 4+                        | 2+   | 1+   | 1+   | —    |

4+ indicates compact, disc-like agglutination, not easily broken; 3+ a compact, disc-like agglutination which breaks up into large clumps; 2+, smaller clumps; 1+, a definite, but finely granular agglutination; — no agglutination visible to the naked eye.

case of non-specific agglutination is a characteristic only of Type I antipneumococcic horse serum.

*Agglutinin Adsorption.*—Using a monovalent Type I antipneumococcic horse serum, tests were made for adsorption of agglutinins for Type I pneumococcus and *Esch. coli* 8-a. Equal parts of the cultures and serum dilutions were mixed and incubated 2 hours in a 40°C. water bath and overnight in the refrigerator.

Readings were made and the mixtures centrifuged. The clear supernatants were transferred in the same order to duplicate agglutination tubes. The serum treated with each culture was then retested for the presence of agglutinins for each organism as before. The results are shown in Tables II and III.

It can be readily observed that, by this procedure, most of the agglutinins for *Esch. coli* 8-a were removed by adsorption with *Esch. coli* 8-a, but those for Type I pneumococcus were relatively unaffected. Similar treatment of the serum with Type I pneumococci effectively removed agglutinins for both organisms.

TABLE II

*Agglutinins for Type I Pneumococcus and Esch. coli 8-a in Type I Antipneumococcic Horse Serum before Adsorption*

| Type I antipneumococcic horse serum | Cultures        | Final dilutions of serum |      |      |      |      |       |
|-------------------------------------|-----------------|--------------------------|------|------|------|------|-------|
|                                     |                 | 1:5                      | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 |
| 576-4                               | Pn I            | 3+                       | 4+   | 4+   | 4+   | 3+   | 1+    |
|                                     | <i>Coli</i> 8-a | 4+                       | 4+   | 4+   | 3+   | 1+   | ±     |

TABLE III

*Agglutinins for Type I Pneumococcus and Esch. coli 8-a in Type I Antipneumococcic Horse Serum after Adsorption*

| Serum adsorbed by | Cultures added to supernatants | Final dilutions of serum |      |      |      |       |       |
|-------------------|--------------------------------|--------------------------|------|------|------|-------|-------|
|                   |                                | 1:10                     | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 |
| Pn I              | Pn I                           | 3+                       | 2+   | —    | —    | —     | —     |
|                   | <i>Coli</i> 8-a                | 3+                       | —    | —    | —    | —     | —     |
| <i>Coli</i> 8-a   | Pn I                           | 2+                       | 3+   | 3+   | 2+   | —     | —     |
|                   | <i>Coli</i> 8-a                | 2+                       | —    | —    | —    | —     | —     |

*Serological Tests with Anti-Coli 8-a Serum.*—Rabbits were immunized with heat-killed vaccines made from *Esch. coli* 8-a. Two courses of fifteen doses each were given. The schedules and amounts were similar to those used in preparing antipneumococcic rabbit serums (3). The rabbits were bled by cardiac puncture 7 days after the last dose in each course of injections. The serums were pooled and preserved by the addition of 0.3 per cent tricresol.

This pooled serum was tested against the homologous organisms, Types I, II, and III pneumococci, Type I SSS, and against a filtrate of the supernatant from a lot of *Esch. coli* 8-a vaccine. In addition, a Type I antipneumococcic horse serum was tested against the *coli* 8-a vaccine filtrate and against Type I SSS.

The agglutination titer of the serum for the homologous organism was 1:5120, and its precipitin titer for the vaccine filtrate was 1:80. No agglutination occurred with Types I, II, and III pneumococci, nor was there any precipitation of Type I SSS. A Type I antipneumococcic horse serum which, in a dilution of 1:160, precipitated Type I SSS (1:10,000 dilution) also precipitated, in a serum dilution of 1:80, a vaccine filtrate of *coli* 8-a.

These results indicate that the cross-reactions between Type I antipneumococcic horse serum and *Esch. coli* 8-a are not reciprocal, and further suggest that a soluble capsular material is elaborated by the colon bacillus.

TABLE IV

*Agglutination of Virulent and Avirulent Strains of Coli 8-a and Stock Strain of Esch. coli by Various Serums*

| Serums                            | Cultures                  | Final dilutions of serums |      |      |      |      |       |       |       |        |  |
|-----------------------------------|---------------------------|---------------------------|------|------|------|------|-------|-------|-------|--------|--|
|                                   |                           | 1:5                       | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 |  |
| Anti- <i>coli</i> 8-a             | Virulent <i>coli</i> 8-a  | 3+                        | 3+   | 3+   | 3+   | 3+   | 2+    | 1+    | 1+    | —      |  |
|                                   | Avirulent <i>coli</i> 8-a | 1+                        | 1+   | 1+   | 1+   | 1+   | 1+    | 1+    | 1+    | 1+     |  |
|                                   | Stock <i>coli</i> K-12    | ±                         | ±    | —    | —    | —    | —     | —     | —     | —      |  |
| Pn I horse serum<br>576-4         | Virulent <i>coli</i> 8-a  | 3+                        | 3+   | 2+   | 1+   | ±    | —     | —     | —     | —      |  |
|                                   | Avirulent <i>coli</i> 8-a | 1+                        | 1+   | 1+   | —    | —    | —     | —     | —     | —      |  |
| Pn I horse serum<br>576-5         | Virulent <i>coli</i> 8-a  | 4+                        | 3+   | 3+   | 1+   | —    | —     | —     | —     | —      |  |
|                                   | Stock <i>coli</i> K-12    | 1+                        | 1+   | 1+   | 1+   | —    | —     | —     | —     | —      |  |
| Pn I and II horse<br>serum 610-17 | Virulent <i>coli</i> 8-a  | 4+                        | 3+   | 2+   | —    | —    | —     | —     | —     | —      |  |
|                                   | Stock <i>coli</i> K-12    | 1+                        | 1+   | 1+   | 1+   | 1+   | —     | —     | —     | —      |  |
| Pn I and II horse<br>serum 615-23 | Virulent <i>coli</i> 8-a  | 3+                        | 2+   | —    | —    | —    | —     | —     | —     | —      |  |
|                                   | Stock <i>coli</i> K-12    | 1+                        | 1+   | 1+   | 1+   | 1+   | —     | —     | —     | —      |  |

*Agglutination Tests with Other Coli Strains.*—For further testing, *Esch. coli* 8-a was transferred through twenty serial passages in 10 per cent Type I antipneumococcic horse serum broth. Although microscopic examination of agar plate cultures of this serum broth culture failed to reveal evidence of rough colony formation, 0.5 cc. of the culture failed to kill mice, and so is designated as an avirulent strain of *coli* 8-a. Agglutination tests tended to indicate that the loss of virulence was accompanied by alterations in reactivity usually associated with strains intermediate between the "S" and "R" forms. An old stock culture of *Esch. coli* (K-12) was also tested for its agglutinability by various serums. The results of these tests are presented in Table IV.

The agglutination reactions of the virulent *coli* 8-a were of the flocculent nature, while those of the avirulent *coli* 8-a and of the K-12 strain were of the finely granular type, and give added evidence that passage of the virulent *coli* 8-a through serum broth had at least partially degraded the organism.

As a matter of interest, the anti-*coli* 8-a rabbit serum was tested against Types A, B, and C Friedländer's bacilli. All tests were completely negative.

#### DISCUSSION

The foregoing data are presented to illustrate another example of the heterogenetic specificity described by Avery, Heidelberger, and Goebel. Their results demonstrated a relationship between Type II pneumococci and Type B Friedländer's bacilli; the present communication establishes a serological connection between Type I pneumococci and an encapsulated strain of *Esch. coli*. The two reports suggest a possible explanation for some of the discrepancies in typing suspected pneumonia sputums by various methods, particularly when anti-pneumococcic horse serums are used in diagnosis.

The occurrence of encapsulated strains of *Esch. coli* is not uncommon. Their existence is mentioned by Ford (4a), and extensive studies of their characteristics were made by Smith and his associates (4b). Precipitin tests with a filtrate of vaccine made from the 8-a strain indicate that the capsular material may be elaborated in soluble form. Such an observation is in conformity with the findings of Tomcsik (5) who prepared purified polysaccharides from encapsulated colon and *aerogenes* bacilli. Work is in progress in attempts to isolate the carbohydrate fraction of *Esch. coli* 8-a.

The source of the colon bacillus herein described is unknown. It is probably of fecal origin as indicated by its inability to utilize citrate (6), and the negative Voges-Proskauer and positive methyl red reactions. The possibility that the organism entered as a contaminant from the mouse cannot be overlooked. Assuming, however, that both pneumococci and colon bacilli were present in the upper respiratory tract of the human subject, it is not unlikely that the colon organisms would obliterate the pneumococci during mouse passage. The substitution of the Neufeld *Quellung* reaction in diagnosing suspected

pneumonia sputums for the mouse passage method would, however, overcome such a difficulty.

It is of interest to note that the lack of serological specificity observed in this study is a characteristic limited to Type I antipneumococcic horse serum. The facts cited suggest that Type I pneumococci contain a reactive substance not possessed by the colon bacillus, and which may be antigenic in horses but not in rabbits. This offers one possible explanation for the greater specificity commonly assumed for rabbit serums used in the Neufeld *Quellung* test.

#### SUMMARY

An encapsulated strain of *Escherichia coli* has been isolated which is hemolytic, pathogenic for mice, and which has served to illustrate further evidence of heterogenetic specificity. The relationship appears to be limited to the serological reactions between the colon organism and Type I antipneumococcic horse serum. Type I antipneumococcic rabbit serum failed to agglutinate the organism and no reactions occurred with Types II and III antipneumococcic horse serums. Serum from rabbits immunized with the colon bacillus agglutinated the homologous organism and precipitated its soluble substance, but failed to cause agglutination of Type I pneumococci or to precipitate Type I pneumococcic polysaccharide. The evidence indicates a connection somewhat analogous to that between Type II pneumococcus and Type B Friedländer's bacillus.

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# THE ANTIGENIC RELATIONSHIP BETWEEN BACILLUS PROTEUS X-19 AND RICKETTSIAE

## III. A STUDY OF THE ANTIGENIC COMPOSITION OF THE EXTRACTS OF BACILLUS PROTEUS X-19

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In two previous papers (1, 2) we have reported upon the presence in *B. proteus* X-19 and in Mexican *Rickettsiae* of a soluble specific substance common to both organisms and responsible for the agglutination of *B. proteus* X-19 in the Weil-Felix reaction.

The present study deals with a further analysis of the antigenic complex of *B. proteus* X-19, and describes, two substances isolated from this organism, one of which reacts with both antityphus serum and anti-*Proteus* serum, the other reacting with the latter only.

The existence of an antigenic factor common to *Rickettsia* and *Proteus* X-19 was contrary to the usual experience that the sera of animals immunized with *Proteus* X-19 failed to agglutinate *Rickettsia* suspensions. The writer with Zia (1), however, explained this discrepancy by showing that Mexican *Rickettsiae* agglutinated in anti-*Proteus* serum provided that the organisms were washed with 0.2 per cent formalin in salt solution, from the peritoneal cavity of rats which had been X-rayed and subsequently inoculated with typhus. The purified formalinized suspensions of *Rickettsiae* thus obtained were heated at 75°C. for 30 minutes.

Lim and Kurotchkin (3), White (4), the writer (2) and, recently, Kemp and Cain (5) have obtained soluble specific substances from *B. proteus* X-19 by the usual methods of preparing specific polysaccharides from bacteria. Our preparations give strong Molish tests, negative biuret reactions, are heat-stable, withstand boiling in alkaline reaction, resist digestion by trypsin and contain 4 per cent nitrogen. It seems likely, therefore, that these extracts belong to the polysaccharide group of antigens. Such extracts have the property of precipitating with the serum of typhus patients, as well as with the homologous serum.

White (4) found that the Seitz filtrates of boiled saline suspensions of *B. proteus* X-19 precipitated with both anti-*Proteus* serum and in the serum of typhus

patients. The same filtrates, when heated at 55°C. with 0.5 per cent NaOH for a few minutes, lost almost entirely their power of reacting with anti-*Proteus* X-19 sera, though retaining that of reacting with typhus serum. He also found that by heating *B. proteus* X-19 with 5 per cent antiformin or 1 per cent NaOH for 2 hours he could obtain extracts which precipitated with the serum of typhus patients, but not with the homologous serum. He explains this fact by considering the *Proteus* X-19 extracts as containing two receptors: the chief, or alkali-labile receptor being responsible for the "O" agglutination of *B. proteus* X-19 in anti-*Proteus* serum, but playing no rôle in the Weil-Felix reaction; and a second, or alkali-stable receptor responsible for the Weil-Felix reaction, but having only a minor or minimal part in the reaction with anti-*Proteus* serum. White did not succeed in isolating such receptors.

The antigen common to *Rickettsiae* and *B. proteus* X-19 we shall refer to as the "X factor," and the antigen reacting only with *Proteus* X-19 antiserum as the "P factor."

### *Materials and Technique*

*B. Proteus* X-19.—The "OX-19 (1)," obtained from Dr. Mooser and preserved in this laboratory for its "O" characteristics during the last 5 years.

*Anti-Proteus* X-19 Serum.—Prepared by immunizing rabbits with broth cultures of *B. proteus* X-19.

*Typhus Human Serum*.—(a) Obtained through the courtesy of Dr. Varela from cases of Mexican typhus. (b) Sera from patients suffering from Brill's disease in Boston.

*Typhus Antiserum*.—Prepared in this laboratory by intravenous injection of horses with formalinized suspensions of *Rickettsiae*.

### *Preparation of B. proteus* X-19 Extracts

1. *Antiformin Method*.—Agar cultures of *B. proteus* X-19 grown in Kolle flasks are washed in saline and centrifuged to remove impurities from the medium, then resuspended in a small volume of water and mixed with four volumes of a 6.25 per cent solution of antiformin, which gives a final concentration of 5 per cent in the mixture. This is shaken for 15 to 30 minutes at room temperature, and acetic acid immediately added until a precipitate forms. After centrifugation, the supernatant fluid is dialyzed for 48 to 72 hours. The dialyzed material (about 1 liter per 50 Kolle flasks) is then concentrated to about one-fifth of its volume by evaporation in the boiling water bath under the electric fan. The concentrated material is treated with one and a half volumes of cooled alcohol and left overnight in the refrigerator. The precipitate thus obtained contains the X factor, which can be further purified by alcohol fractionation. To the alcoholic supernatant, after removing the X-containing substance, is added an excess of alcohol and some

sodium acetate solution, until a new, heavy precipitate forms. This precipitate contains most of the P factor.

The purification of the X factor is carried out by fractionation with alcohol. This is done by addition of alcohol until a precipitate is obtained. The precipitate is removed by centrifuging. More alcohol is added to the supernatant until a new precipitate forms. This again is removed and the process repeated until no more precipitate is obtained. The precipitate is selected which gives the highest precipitinogen titre when tested with typhus serum. The purification of each lot of X factor requires different quantities of alcohol, according to the amount of electrolytes present.

The X and P factors both require the presence of electrolytes in order to be precipitated by alcohol, but the P substance has to have an excess of both electrolyte and alcohol.

Further purification of the X and P factors may be carried out by shaking these extracts with chloroform in a manner similar to that recommended by Sevag (6). The shaking of the extracts with chloroform for 24 hours at a time has to be repeated until no more precipitate forms. The chloroform precipitates the protein as a jelly-like substance easily separated by centrifugation.

The final nitrogen content of the X substance was 4 per cent, and that of the P substance 0.9 per cent. Some P factor remaining in the X extracts can be inactivated by heating the extracts at the boiling point in a N/50 concentration of NaOH.

2. *Grinding Method*.—Agar cultures of *B. proteus* X-19 were washed in saline, centrifuged, and resuspended in as small a volume as possible. The emulsion was ground for 72 hours in a pyrex tube 20 cm. long by 4 cm. in diameter, containing 200, 1/4 inch stainless steel balls. The corked tube was rotated at 280 R.P.M. in a machine similar to that described by Mak (7).

After grinding, the material was washed from the tube with saline and centrifuged at high speed. The bacterial extract was submitted to chloroform purification until no more precipitate formed. Successively increasing volumes of alcohol were added to the fluid, and those fractions containing the X and P substances in the greatest concentration were detected by means of precipitation tests with typhus and anti-*Proteus* sera. The X factor was precipitated by addition of up to three volumes of alcohol. The precipitation of the P factor required a considerably larger proportion of alcohol.

3. Similar extracts were prepared by tryptic digestion and by freezing and thawing of agar cultures of *B. proteus* X-19.

## EXPERIMENTAL

Table I shows the results of precipitation tests with typhus and anti-*Proteus* sera and X and P factors obtained by the antiformin method. Similar results were obtained with extracts prepared by grinding and by freezing and thawing *B. proteus* X-19. The extracts

used as X factor still contained some P factor; but the P extracts were serologically pure.

As is seen in Table I, the X factor reacts with both typhus and anti-*Proteus* sera, while the P factor reacts only with anti-*Proteus* serum.

The results of our own experiments confirm in principle the presence in crude *Proteus* X-19 extracts of two types of receptors. They differ from those of White in that we found that treating with alkali did not

TABLE I

*Precipitation of Typhus and Anti-Proteus Sera with X and P Receptors*

| Sera                           | X factor (unheated) |          |          |           |             | P factor |          |          |           |             |
|--------------------------------|---------------------|----------|----------|-----------|-------------|----------|----------|----------|-----------|-------------|
|                                | 1/1,000             | 1/10,000 | 1/50,000 | 1/100,000 | 1/1,000,000 | 1/1,000  | 1/10,000 | 1/50,000 | 1/100,000 | 1/1,000,000 |
| Anti- <i>Proteus</i> rabbit    | ++++                | ++++     | +++      | ++        | ±           | ++++     | ++++     | ++++     | +++       | +           |
| Boston case of Brill's disease |                     | +++      | ++       | +         | ±           | —        | —        | —        | —         | —           |
| Mexican typhus (convalescent)  |                     | +++      | ++       | ±         | —           | —        | —        | —        | —         | —           |
| Antityphus horse               | +++                 | ++       | ++       | +         | ±           | —        | —        | —        | —         | —           |

inactivate the X factor in regard to its reaction with anti-*Proteus* serum. In our hands, boiling for 1 hour in N/50 NaOH destroyed the P factor completely, while the X factor retained its activity toward both typhus and anti-*Proteus* sera.

Table II illustrates the results obtained by heating X and P factors diluted to 1/2,000 and adding enough NaOH to make a N/50 concentration.

In order to compare our results with those of White, we treated our X factor with 1 per cent NaOH at different temperatures and for different periods of time. We found that heating 1/1,000 dilutions of X factor at 55°C. for 2 hours did not destroy its activity toward

anti-*Proteus* serum, and after such X substance, in alkali, had been submitted to autoclaving at 15 pounds for 30 minutes, it was still possible to detect precipitinogen for the homologous serum.

We have not been able to obtain powerful extracts by following White's techniques. These had very low titres compared with our own extracts, but although weak they showed precipitating power for anti-*Proteus* serum.

The differences so far described between X and P factors can be further substantiated by the results of the absorption of precipitins from typhus and anti-*Proteus* sera, with pure X and P factors. As is shown in Table III, typhus serum and anti-*Proteus* serum were ab-

TABLE II  
*Comparative Precipitation Tests of Alkali-Treated X and P Substances and Untreated Controls*

|                            | Untreated antigens |          |           | Heated antigens in N/50 NaOH |          |           |          |
|----------------------------|--------------------|----------|-----------|------------------------------|----------|-----------|----------|
|                            | 1/10,000           | 1/50,000 | 1/250,000 | 1/10,000                     | 1/50,000 | 1/250,000 |          |
| Typhus human serum         | —                  | —        | —         | —                            | —        | —         | P factor |
| Anti- <i>Proteus</i> serum | ++++               | ++++     | +++       | —                            | —        | —         | P factor |
| Typhus human serum         | ++                 | +        | ±         | ++                           | +        | ±         | X factor |
| Anti- <i>Proteus</i> serum | ++++               | ++++     | +++       | ++++                         | +++      | ++        | X factor |

sorbed with P and X factors, the latter after purification with alkali, and controls absorbed with whole X-19 extracts.

From these experiments it appears that two soluble specific substances can be isolated from *B. proteus* X-19, one of which is stable on heating in alkaline reaction and precipitates antityphus and anti-*Proteus* sera. The second substance is alkali-labile and precipitates exclusively with anti-*Proteus* serum.

When anti-*Proteus* serum is absorbed with X factor, the serum retains its power of precipitating with P substance and agglutinates *B. proteus* X-19, without appreciable change in titre. As was shown in a former paper, the absorbed anti-*Proteus* serum loses its property of agglutinating *Rickettsia* suspensions.

TABLE III  
Absorption of Proteus X-19 Antiserum and Typhus Serum with X and P Receptors

| Sera  | Precipitations with |               |                       | Agglutinations with <i>B. proteus</i> X-19 |      |       |       |       |         |         |
|---|---------------------|---------------|-----------------------|--|------|-------|-------|-------|---------|---------|
|   | P<br>1/15,000       | X<br>1/10,000 | Whole X-19<br>extract | 1/40                                       | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | 1/2,560 |
| Anti- <i>Proteus</i> unabsorbed                     | ++++                | ++            | ++++                  | ++++                                       | ++++ | ++++  | ++++  | ++++  | ++      | —       |
| Anti- <i>Proteus</i> absorbed with<br>P 1/2,000     | —                   | ++            | ++                    | ++++                                       | ++++ | +     | —     | —     | —       | —       |
| Anti- <i>Proteus</i> absorbed with<br>X 1/2,000     | ++++                | ±             | ++++                  | ++++                                       | ++++ | ++++  | ++++  | ++++  | ++      | —       |
| Anti- <i>Proteus</i> absorbed with<br>whole extract | —                   | —             | —                     | —  | —    | —     | —     | —     | —       | —       |
| Typhus human unabsorbed*                            | —                   | ++            | +                     | ++++                                       | ++++ | ++++  | ++++  | ++++  | +       | ±       |
| Typhus human absorbed with<br>P 1/2,000             | —                   | ++            | +                     | ++++                                       | ++++ | ++++  | ++++  | ++++  | ++      | ±       |
| Typhus human absorbed with<br>X 1/2,000             | —                   | —             | —                     | —  | —    | —     | —     | —     | —       | —       |
| Typhus human absorbed with<br>whole extracts        | —                   | —             | —                     | —  | —    | —     | —     | —     | —       | —       |

\* Similar results were obtained with the serum of a horse treated by intravenous injections of formalized Mexican *Rickettsiae*.

When typhus serum is treated with X substance, the serum loses completely its agglutinating power for *B. proteus* X-19.

The absorption of anti-*Proteus* serum with P reduces in a considerable proportion its agglutinating titre for *B. proteus* X-19, but there remain precipitins for the X factor. Antityphus serum is not influenced by P.

Anti-*Proteus* serum absorbed with whole X-19 extracts loses its agglutinins for *B. proteus* X-19, as well as precipitins for the isolated substances. The effect of the whole extracts on typhus serum is identical with that of the isolated X factor.

#### SUMMARY AND CONCLUSIONS

Two substances differing in immunological behavior as well as in certain chemical properties have been isolated from soluble extracts of *B. proteus* X-19. Both substances appear to be polysaccharides.

The first substance is precipitated from X-19 crude extracts by a relatively low percentage of alcohol and electrolytes (from one to two and a half volumes of alcohol). When purified as far as possible, it gives a negative biuret reaction, a positive Molish and has a nitrogen content of 4 per cent. This material, which we call X factor, has the immunological properties of the common antigenic factor in *Proteus* X-19 and typhus *Rickettsiae*, described elsewhere. It has the property of precipitating with typhus serum as well as anti-*Proteus* serum, even after treatment with hot alkali.

The second substance we call P factor, suggesting a material which is proper to *Proteus* X-19 and has nothing to do with the Weil-Felix reaction. It is obtained from the crude extracts of *B. proteus* X-19 by treating the fluids from which the X factor has been removed with an excess of alcohol (seven to ten volumes, according to electrolytes in solution). The purified material shows a nitrogen content of a little less than 1 per cent, gives a negative biuret and a positive Molish reaction. The P factor produces precipitates with anti-*Proteus* serum in considerable dilution, but has no effect on typhus serum. It is quickly destroyed on treating with alkali, a fact in accordance with the results already cited, which were obtained by White with whole extracts of *B. proteus* X-19.

The duality of the X-19 extracts seems to be explained by the isolation of two immunologically different factors; one which is alkali-labile



and which is proper to *B. proteus* X-19; and the other which is alkali-stable and is the common antigenic factor in *Proteus* X-19 and typhus *Rickettsiae*.

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# A MODIFIED METHOD OF OBTAINING LARGE AMOUNTS OF RICKETTSIA PROWAZEKI BY ROENTGEN IRRADIATION OF RATS

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In 1932, Zinsser and Castaneda (1) described a method of obtaining large amounts of Mexican typhus *Rickettsiae*, utilizing the peritoneal suspension obtained by washing the scraped peritoneum of white male rats with a 0.2 per cent formalinized citrate solution.

In order to lower the resistance of the animals, they were irradiated previous to inoculation, using a high voltage Roentgen therapy apparatus. The following factors were employed: 170 kv. peak constant potential (equivalent to 190 kv. peak pulsating current); 80 cm. distance;  $\frac{1}{2}$  mm. copper filter; 8 ma. With this setting, 10 Roentgen units per minute, measured in air, was obtained. The most suitable exposure for the rats was found to be 600 r; that is, a 60 minute treatment time.

Immediately after irradiation, the rats were inoculated intraperitoneally with a suspension of Mexican typhus virus from a guinea pig's infected tunica vaginalis, which was not richer in *Rickettsiae* than the average obtained in routine transfer.

By this technique, the authors mentioned developed a sure and practical method of obtaining a highly concentrated suspension of specific Mexican typhus *Rickettsiae* which they used principally in the preparation of typhus vaccine and specific immune horse serum. The use of this vaccine in Mexico by Varela and Parada (2) and by Sanchez Casco (3) and the study of the therapeutic value of the serum by the Mexican Commission headed by Dr. Landa have given such promising results that it would seem advisable to recommend the extension of this method to those countries affected by the disease.

\* At the time of doing this work, Dr. Macchiavello held a fellowship from the John Simon Guggenheim Memorial Foundation.

Cross-immunity tests on animals, using the European virus, have demonstrated a certain degree of protection against the Old World form of the disease, and it would seem justifiable to undertake these cross-immunity tests on human beings in those countries affected by European typhus.

#### EXPERIMENTAL

Zinsser and Castaneda appreciated the difficulties of their method when a high voltage Roentgen unit was not available for irradiation of the rats prior to inoculation. Moreover, extensive experimental efforts at the Harvard laboratory to find substitutes for the radiation method merely demonstrated that, for the present, this method was the only available one which gave practically utilizable results. It was desirable, therefore, to attempt to work out a technique of radiation for typhus vaccine production in which standard radiographic machines, everywhere available, could be employed. The experiments herewith submitted represent efforts to accomplish this at the Harvard laboratories. The machine used was equipped with a full wave mechanical rectifier of the cross-arm type. A broad focus universal tube, made by the General Electric X-Ray Corporation, was employed. The following factors, which may easily be obtained on any good radiographic machine, were kept constant throughout all experiments: 120 kv. peak (sphere gap measurement); 4 ma.; 40 cm. distance. The only variable was the filter. In the first series of experiments,  $\frac{1}{8}$  mm. of copper was employed. In the second series, no filter was used. The Roentgen unit measurement with  $\frac{1}{8}$  mm. of copper in place was 16.1 r per minute, and with no filter it was 92.0 r per minute. The other points of bacteriological technique did not differ from those described by Zinsser and Castaneda.

In Table I we have summarized the results of the experiments, using  $\frac{1}{8}$  mm. copper filter. We obtained a quantity of *Rickettsiae*, but not enough for vaccine purposes.

The experiments using no filter are recorded in Table II and are much more satisfactory. It will be seen from that table that doses of unfiltered radiation below 3,000 r are of little value. The most effective doses have been from 3,500 to 3,600 r, which, with the factors given above, means a treatment time of 38 to 40 minutes. With this dosage, most of the rats live until the 5th day, which has been found experimentally to be the best time at which to kill the animals, since the tunica and peritoneum are then richest in *Rickettsiae*. With doses above 3,600 r, there is a light mortality of the rats before the 5th day.

We must call attention to the fact that the effect of Roentgen irradiation is not always uniform. There is some small variation in animals of the same lot, and one lot varies slightly in comparison with another. Moreover, there is a seasonal variation which has been noted in work at the Harvard laboratories. In the hot months, the peritoneal exudates of radiated rats are often less rich in organisms. There is no adequate explanation of this seasonal fluctuation at present

TABLE I

*Relation between Roentgen Exposure, with  $\frac{1}{8}$  Mm. Copper Filter, and the Amount of Rickettsiae in the Peritoneal Suspension of Exudate and Scraping, in White Male Rats*

| Rat No. | Time of exposure | Total r units | Amount of Rickettsiae |        | Died (length of time after X-ray) | Killed (length of time after X-ray) | Date of radiation |
|---------|------------------|---------------|-----------------------|--------|-----------------------------------|-------------------------------------|-------------------|
|         |                  |               | Peritoneum            | Tunica |                                   |                                     |                   |
|         | min.             |               |                       |        |                                   | days                                | 1934              |
| 1 L1R1  | 31               | 500           | +                     | ++     |                                   | 5                                   | Nov. 19           |
| 2 L3R4  | 50               | 800           | +++                   | +      |                                   | 5                                   | Dec. 5            |
| 3 L3R5  | 50               | 800           | +                     | +      |                                   | 5                                   | " 5               |
| 4 L3R6  | 50               | 800           | +                     | ++     |                                   | 5                                   | " 5               |
| 5 L1R3  | 62               | 1,000         | 0                     |        | 2 hrs.                            |                                     | Nov. 19           |
| 6 L2R4  | 62               | 1,000         | ++                    | ++     |                                   | 5                                   | " 26              |
| 7 L2R5  | 62               | 1,000         | +                     | +      | 4 days                            |                                     | " 26              |
| 8 L2R6  | 62               | 1,000         | ++                    | +      |                                   | 5                                   | " 26              |
| 9 L1R5  | 93               | 1,500         | +                     | ++     | 4 days                            |                                     | " 19              |

0 = *Rickettsiae* not present.

+

= present in small quantity.

++ = not very rich (insufficient for vaccine).

+++ = very rich.

The results of irradiation using  $\frac{1}{8}$  mm. copper filter. Some *Rickettsiae* are obtained but not enough for vaccine purposes.

but experiments carried out in the department have led us to suspect there is an intimate relationship between this phenomenon and the high leukocytosis shown by rats in the summer months. These fluctuations, however, are confined to narrow limits and do not make the method unreliable or detract from its usefulness.

Table III shows the relationship between the leukopenia produced by irradiation and the richness in *Rickettsia* of the peritoneal exudate

TABLE II

*Relation between Roentgen Exposure without Filler and the Amount of Rickettsiae in the Peritoneal Suspension of Exudate and Scraping, in White Male Rats.*

*All Factors Constant Except Time of Exposure*

| Rat No. | Time of exposure | Total r units | Amount of <i>Rickettsiae</i> |        | Died (length of time after X-ray) | Killed (length of time after X-ray) | Date of radiation |
|---------|------------------|---------------|------------------------------|--------|-----------------------------------|-------------------------------------|-------------------|
|         |                  |               | Peritoneum                   | Tunica |                                   |                                     |                   |
|         | <i>min.</i>      |               |                              |        |                                   | <i>days</i>                         | <i>1934</i>       |
| 1 L4R4  | 5½               | 500           | 0                            | 0      |                                   | 7                                   | Dec. 17           |
| 2 L4R5  | 11               | 1,000         | 0                            | +      |                                   | 7                                   | " 17              |
| 3 L4R6  | 16½              | 1,500         | +                            | +      |                                   | 6                                   | " 17              |
| 4 L5R4  | 22               | 2,000         | +                            | ++     |                                   | 5                                   | " 31              |
| 5 L5R5  | 27½              | 2,500         | +++                          | ++     |                                   | 5                                   | " 31              |
|         |                  |               |                              |        |                                   |                                     | <i>1935</i>       |
| 6 L6R1  | 27½              | 2,500         | +                            | +      | 3 days                            |                                     | Jan. 10           |
| 7 L8R1  | 27½              | 2,500         | +++                          | +++    |                                   | 5                                   | " 16              |
| 8 L8R2  | 27½              | 2,500         | +                            | +      |                                   | 5                                   | " 16              |
| 9 L8R3  | 27½              | 2,500         | +++                          | +++    |                                   | 5                                   | " 16              |
| 10 L8R4 | 27½              | 2,500         | ++                           | +++    |                                   | 5                                   | " 16              |
|         |                  |               |                              |        |                                   |                                     | <i>1934</i>       |
| 11 L5R6 | 33               | 3,000         | ++                           | ++     |                                   | 5                                   | Dec. 31           |
|         |                  |               |                              |        |                                   |                                     | <i>1935</i>       |
| 12 L6R2 | 33               | 3,000         | +                            | +      | 3 days                            |                                     | Jan. 10           |
| 13 L6R3 | 33               | 3,000         | +                            | +      | 3 "                               |                                     | " 10              |
| 14 L9R1 | 33               | 3,000         | 0                            | +      |                                   | 5                                   | " 31              |
| 15 L9R2 | 33               | 3,000         | +                            | +      |                                   | 5                                   | " 31              |
| 16 L9R3 | 33               | 3,000         | ++                           | ++     |                                   | 5                                   | " 31              |
| 17 L9R4 | 33               | 3,000         | ++                           | ++     |                                   | 5                                   | " 31              |
| 18 L7R2 | 38½              | 3,500         | ++                           | ++     | 60 hrs.                           |                                     | " 12              |
| 19 L7R3 | 38½              | 3,500         | +                            | +      | 70 "                              |                                     | " 12              |
| 20 L9R5 | 38½              | 3,500         | ++++                         | ++++   |                                   | 5                                   | " 31              |

0 = *Rickettsiae* not present.

+ = present in small quantity.

++ = useful for vaccine but not very rich.

+++ = very rich.

++++ = extraordinarily rich.

Each lot of rats was properly controlled with irradiated normal rats. Moreover, similar lots were inoculated with the same virus after irradiation by the original high voltage method. A dose of 3,500 r with the modified low voltage method gives the same results as 600 r by the original high voltage method. Failures are not due to variation in radiation but are the result of undetermined factors; perhaps variations in individual resistance of the animals, or unexpected variation of the virulence of the virus, etc.

TABLE II—*Concluded*

| Rat No.  | Time of exposure | Total r units | Amount of <i>Rickettsiae</i> |        | Died (length of time after X-ray) | Killed (length of time after X-ray) | Date of radiation |
|----------|------------------|---------------|------------------------------|--------|-----------------------------------|-------------------------------------|-------------------|
|          |                  |               | Peritoneum                   | Tunica |                                   |                                     |                   |
|          | <i>min.</i>      |               |                              |        |                                   | <i>days</i>                         | <i>1935</i>       |
| 21 L9R6  | 38½              | 3,500         | 0                            | 0      | 6 hrs.                            |                                     | Jan. 31           |
| 22 L9R7  | 38½              | 3,500         | ++++                         | ++++   |                                   | 5                                   | " 31              |
| 23 L9R8  | 38½              | 3,500         | 0                            | 0      | 48 hrs.                           |                                     | " 31              |
| 24 L10R1 | 38½              | 3,500         | +++                          | +++    |                                   | 5                                   | Feb. 12           |
| 25 L10R2 | 38½              | 3,500         | ++                           | +++    |                                   | 5                                   | " 12              |
| 26 L10R3 | 38½              | 3,500         | ++++                         | +++    |                                   | 5                                   | " 12              |
| 27 L10R4 | 38½              | 3,500         | ++                           | +++    |                                   | 5                                   | " 12              |
| 28 L10R5 | 38½              | 3,500         | ++                           | ++     | 4 days                            |                                     | " 12              |
| 29 L10R6 | 38½              | 3,500         | +                            | +      | 48 hrs.                           |                                     | " 12              |
| 30 L10R7 | 38½              | 3,500         | ++                           | +++    |                                   | 5                                   | " 12              |
| 31 L10R8 | 38½              | 3,500         | +                            | ++     |                                   | 5                                   | " 12              |
| 32 L11R1 | 38½              | 3,500         | +++                          |        |                                   | 5                                   | Apr. 3            |
| 33 L11R2 | 38½              | 3,500         | +++                          |        |                                   | 5                                   | " 3               |
| 34 L11R3 | 38½              | 3,500         | ++++                         |        |                                   | 5                                   | " 3               |
| 35 L12R1 | 40               | 3,666         | +++                          |        |                                   | 5                                   | " 16              |
| 36 L12R2 | 40               | 3,666         | +++                          |        |                                   | 5                                   | " 16              |
| 37 L12R3 | 40               | 3,666         | ++++                         |        |                                   | 5                                   | " 16              |
| 38 L12R4 | 40               | 3,666         | +                            |        |                                   | 5                                   | " 16              |
| 39 L12R5 | 40               | 3,666         | +                            |        | 4 days                            |                                     | " 16              |
| 40 L12R6 | 40               | 3,666         | ++++                         |        |                                   | 5                                   | " 16              |
| 41 L13R1 | 40               | 3,666         | +++                          |        |                                   | 5                                   | " 16              |
| 42 L13R2 | 40               | 3,666         | ++                           |        |                                   | 5                                   | " 16              |
| 43 L13R3 | 40               | 3,666         | +++                          |        |                                   | 5                                   | " 16              |
| 44 L13R4 | 40               | 3,666         | +++                          |        |                                   | 5                                   | " 16              |
| 45 L7R4  | 44               | 4,000         | +                            | +      | 2 days                            |                                     | Feb. 12           |
| 46 L7R5  | 44               | 4,000         | +                            | ++     | 60 hrs.                           |                                     | " 12              |
| 47 L7R6  | 44               | 4,000         | ++                           | ++     | 72 "                              |                                     | " 12              |
| 48 L7R7a | 44               | 4,000         | 0                            | 0      | 73 "                              |                                     | Apr. 24           |
| 49 L7R8a | 44               | 4,000         | 0                            | +      | 76 "                              |                                     | " 24              |
| 50 L14R3 | 44               | 4,000         | +                            | 0      | 80 "                              |                                     | " 24              |
| 51 L14R4 | 44               | 4,000         | 0                            | 0      | 50 "                              |                                     | " 24              |
| 52 L14R5 | 44               | 4,000         | 0                            | +      | 3 days                            |                                     | " 24              |
| 53 L14R6 | 44               | 4,000         | +                            | 0      | 3 "                               |                                     | " 24              |
| 54 L7R7  | 49½              | 4,500         | 0                            | 0      | 72 hrs.                           |                                     | Jan. 12           |
| 55 L7R8  | 49½              | 4,500         | 0                            | +      | 74 "                              |                                     | " 12              |
| 56 L7R9  | 49½              | 4,500         | +                            | +      | 76 "                              |                                     | " 12              |

and scrapings. It appears that this relationship is constant in the rats killed on the 5th day when the infection has reached its peak, and is not constant in the animals which die before the 5th day. Thus,

TABLE III  
Relation between the Decrease of Leukocytes and the Amount of Rickettsiae in White Male Roentgen Radiated Rats (without Reference to Rats Dead before 5 Days)

| Rat No.                   | 1     | 2     | 3     | 4     | 5     | 7     | 8     | 9     | 10    |
|---------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| r units.                  | 500   | 1,000 | 1,500 | 2,000 | 2,500 | 2,500 | 2,500 | 2,500 | 2,500 |
| W.B.C. before X-ray       | 20.0  | 18.5  | 15.4  | 11.2  | 10.4  | 20.6  | 15.4  | 18.1  | 9.0   |
| 1 day after X-ray         | 8.0   | 7.0   | 3.1   | 4.5   | 7.0   | 4.1   | 4.6   | 2.6   | 6.5   |
| 2 days "                  | 3.6   | 5.5   | 5.4   | 3.3   | 3.0   | 2.4   | 3.9   | 1.2   | 3.0   |
| 3 " "                     | 4.5   | 1.7   | 1.2   | 0.7   | 0.1   | 0.2   | 0.6   | 0.1   | 0.4   |
| 4 " "                     | 2.2   | 2.3   | 1.2   | 1.5   | 0.3   | 0.6   | 0.8   | 0.9   | 1.2   |
| 5 " "                     | 8.0   | 8.6   | 3.3   | 3.5   | 0.6   | 1.3   | 1.0   | 1.6   | 4.5   |
| 6 " "                     | 15.0  | 10.1  |       |       |       |       |       |       |       |
| 7 " "                     | 22.0  | 12.8  |       |       |       |       |       |       |       |
| Rickettsiae in peritoneum | 0     | 0     | +     | +     | +++   | +++   | +     | +++   | ++    |
| Rat No.                   | 11    | 14    | 15    | 16    | 17    | 20    | 22    | 24    | 25    |
| r units.                  | 3,000 | 3,000 | 3,000 | 3,000 | 3,000 | 3,500 | 3,500 | 3,500 | 3,500 |
| W.B.C. before X-ray       | 14.6  | 9.8   | 14.0  | 21.0  | 80.0  | 17.6  | 12.8  | 9.7   | 11.2  |
| 1 day after X-ray         | 8.1   | 2.7   | 2.1   | 4.8   | 48.5  | 3.2   | 7.3   | 1.5   | 5.0   |
| 2 days "                  | 3.7   | 1.6   | 3.4   | 2.5   | 8.3   | 3.2   | 0.2   | 1.1   | 2.4   |
| 3 " "                     | 0.2   |       |       |       |       |       |       | 0.2   | 0.5   |
| 4 " "                     | 0.4   | 3.2   | 1.5   | 2.5   | 2.6   | 0.2   | 1.6   | 0.5   | 1.0   |
| 5 " "                     | 0.3   | 24.0  | 1.9   | 1.4   | 1.5   | 0.2   | 0.6   | 0.5   | 2.6   |
| Rickettsiae in peritoneum | ++    | 0     | +     | ++    | ++    | +++   | +++   | +++   | ++    |

| Rat No.                          | 26    | 27    | 30    | 31    | 32    | 33    | 34    | 35    | 36    | 37    |
|----------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| r units                          | 3,500 | 3,500 | 3,500 | 3,500 | 3,500 | 3,500 | 3,500 | 3,666 | 3,666 | 3,666 |
| W.B.C. before X-ray              | 16.2  | 14.6  | 9.0   | 8.8   |       |       |       |       |       |       |
| 1 day after X-ray                | 6.8   | 6.5   | 3.0   | 3.8   |       |       |       |       |       |       |
| 2 days " "                       | 3.0   | 3.8   | 1.6   | 1.8   |       |       |       |       |       |       |
| 3 " " "                          | 1.2   | 3.0   | 0.4   | 1.3   |       |       |       |       |       |       |
| 4 " " "                          | 2.2   | 2.0   | 1.2   | 1.1   | 0.3   | 0.2   | 0.2   | 0.1   | 0.15  | 0.05  |
| 5 " " "                          | 1.0   | 0.75  | 0.2   | 2.2   | +     | ++    | +++   | +++   | +++   | +++   |
| <i>Rickettsiae</i> in peritoneum | ++++  | ++    | ++    | +     | +++   | +++   | +++   | +++   | +++   | +++   |

Leukocytes in thousands.

This shows the distinct relationship between the leukopenia and the richness of the *Rickettsia* in the exudate of the rat peritoneum. The desired leukopenia may be produced by proper irradiation. Perhaps leukopenia is not the only factor necessary for obtaining massive infection of the serosa. It is only one manifestation of the grave shock to the organism. If the shock is too intense the rats die without a fall of leukocytes. If the agranulocytosis is too pronounced the rats die before the infection has spread to the peritoneum.



the problem of irradiating rats is reduced to obtaining rapid leukopenia of less than 1,000 white blood cells per c. mm. with a survival of the animals for 5 days, which is the optimum time for obtaining the best yields of peritoneal *Rickettsia*. Our results are essentially the same as those obtained by Zinsser and Castaneda, but our improvement presents the advantage of being available to any laboratory possessing a good radiographic machine. We are convinced that an expensive high voltage unit is not essential. We are able to obtain a larger quantity of typhus organisms than by any other method. The irradiated rats produce a *Rickettsia* suspension richer than the best typhus tissue culture.

In addition to the changes of X-ray dosage and time of exposure by which we have adapted the rat radiation to standard radiographic machines, we have found from cooperative observations in the Harvard laboratories that results even more regular in yield of *Rickettsiae* can be obtained if the radiation is carried out fractionally by two separate exposures. In this method, the animals are first radiated as described for 20 minutes. This is followed after 48 hours by a second exposure of 30 minutes, following which the animals are intraperitoneally inoculated as usual. This modification has yielded satisfactory results even during the hot months, when for reasons not yet fully determined the *Rickettsia* yield has usually been poor.

#### CONCLUSIONS

The radiation method described by Zinsser and Castaneda for obtaining large amounts of *Rickettsia* has been carried out successfully with an ordinary radiographic machine. This allows the extension of the method to those communities which do not possess a high voltage Roentgen therapy unit as originally employed.

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## RABBIT POX

### IV. SUSCEPTIBILITY AS A FUNCTION OF CONSTITUTIONAL FACTORS

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A devastating epidemic of rabbit pox has been reported in a previous paper of this series (1). In that report, particular attention was devoted to an analysis of evidence concerning the health and functional efficiency of the colony during the period preceding the epidemic as factors of epidemiological significance.

The composition of populations is of equal interest from an epidemiological point of view. Tests of susceptibility to infection may be carried out under controlled experimental conditions, and a great deal of valuable information has been obtained in this way. In the present instance, however, an excellent opportunity was offered for a study of differences in susceptibility to natural infection on the one hand, and of the epidemiological significance of populations on the other, by the spontaneous occurrence of an epidemic in a population composed of known elements. The object of the present paper is to report the results of a study of this kind based largely on an analysis of mortality data with respect to age, sex, race, genetic constitution and physiological status.

#### *Composition of Population*

In previous papers, reference has been made to the fact that the colony was organized for the study of problems in constitution and that at the time of the epidemic it was composed of pure bred and hybrid stocks representative of a number of standard breeds and crosses of various kinds. Detailed descriptions and classifications of animals cannot be given, but as this paper is concerned with the differential response of various elements of the population to an epidemic infection, certain information concerning the population is essential to an interpretation of the results obtained.

*Classification.*—For present purposes, the population of the colony may be divided into three main groups designated as pure bred, hybrid and factor stocks.

This classification is based on racial and genetic considerations, and the divisions are overlapping to the extent that the factor stocks contained both pure bred and hybrid animals.

The pure bred lines represented the basic elements of the population. These were selected originally with a view to building up a population composed of representative types, including animals of diverse and of related racial origins. With few exceptions, the foundation stocks were apparently strong and vigorous animals and conformed to standard specifications for the respective breeds. As the work with these animals progressed, it had been necessary to eliminate most of the larger breeds or so called giant types on account of a lack of space for the proper accommodation of such animals, so that at the time of the epidemic, the colony contained stocks of 15 pure breeds, all of standard weight classes with the exception of a few Belgians which were of the so called heavy weight class. Most of the Belgians were, however, of the standard class.

In general, hybrid and factor stocks were developed from pure bred parents by a process of selective breeding for the purpose of separating and fixing characters which were of interest from the point of view of problems in constitution. These included both physical and functional variations, some of which were apparently harmless while others were invariably lethal when pure and produced definite functional disorders even in heterozygous individuals. The group designated as hybrids included all animals of mixed breed. Some of these had been tested and were known to transmit certain constitutional abnormalities, others were not transmitters, and still others were untested or insufficiently tested to permit of a more accurate classification. The factor stocks included known transmitters of constitutional abnormalities, whether pure bred or hybrid, but only those concerned with functional disorders are of interest in the present connection.

It should also be pointed out that while certain genetic peculiarities had been recognized and investigated to some extent, a complete survey of the colony had not been made and there were doubtless many other constitutional peculiarities which were unknown.

*Pure Breeds.*—The pure bred stocks were represented by the following standard breeds: American Blue, Belgian Hare, Blue Beveren, Chinchilla, Dutch, English, Havana, Himalayan, Lilac, Sable and Silver Marten, Polish, Sable, French Silver or *Argenté de Champagne*, Black and Tan and Blue and Tan, and Rex.

Pure bred stocks are animals that have been bred for a number of generations from established lines in conformity with specifications laid down by representative breeders and fanciers associations. The purity, or homogeneity, of a breed depends largely upon its origin, the age of the breed and the modifications to which it has been subjected. Some breeds are, therefore, much more clearly defined and more homogeneous than others.

The characteristics of breeds are indicated in the so called standards of perfection. In general, these include specifications as to size, color, character of coat and physical conformation, with emphasis on those characters which are most important or most distinctive. In the case of fur breeds, color and quality of

coat are stressed; with the meat breeds, size and age of maturity are the important factors, while with the so called fancy breeds, type and color marking are of foremost importance.

Each of these points possesses some biological significance. It is well to remember, however, that a coat of a given color or character can be used to clothe an animal of any type and while in some instances the coat is a mark of distinction and serves as an indication of racial origin, in others it has no necessary significance. For present purposes, the breeds listed above may be considered from the point of view of size and type relations on the one hand, and racial origins and integrity on the other.

With respect to type, domestic rabbits may be divided into a long slender type and a short compact type with numerous intermediate forms and wide variations in size or weight classifications, as in man. The Belgian Hare and the Dutch are typical of the two main classes, the one long and racy, the other short and cobby. These breeds are products of a period during which fancy furnished the prevailing ideals of perfection. They were show types of animals and of the breeds mentioned above, the English, Black and Tan, Himalayan and Polish are examples of animals in which the conceptions of type and color marking furnished the basic ideals of perfection. The Belgian, English and Himalayan form a series of diminishing size and raciness with fair preservation of the slender type, while the Dutch, Black and Tan and Polish are animals of the compact type but all are of comparatively small size. The Polish is the smallest of all rabbits and some strains are even more cobby than the Dutch. In general, the fancy breeds have been bred true to type and have been subjected to comparatively little experimental modification in recent years.

Recently, utility has dictated the lines of development of domestic rabbits. Extremes of type and the perpetuation of distinctive types have given place to the more conservative intermediate types with size as a major consideration. Meat and fur, or weight and coat, have been the dominant considerations in the development and perfection of breeds in recent years. Other animals listed above come under this general classification. Most of them are of medium size, but the American Blue and Blue Beveren might be described as large in comparison with the smaller Chinchillas, Havanas, Sables, Martens and Lilacs. The French Silver is intermediate and not a product of recent development. From an epidemiological point of view, the chief interest in these breeds is one of racial relations.

*Racial Origins and Relations.*—It is unfortunate that full and accurate information concerning the origin of domestic breeds of rabbits is not available. Little is known concerning the older breeds and accounts differ, or are rather vague, as to the origin of some of the newer breeds. In some instances, origins and relations are traceable through distinctive features of coat color and character. Thus, all chain spotted breeds are genetically related as are those with the Black and Tan pattern regardless of other differences (2).

Of the breeds listed above, the French Silvers and the Himalayans are probably

the oldest; they have been bred for a century or more. Other long established breeds include the English, Dutch, Polish, Tans and Belgians, all of which have been bred for 50 years or more in accordance with well defined standards of perfection. The Beverens and Havanas appeared about 1898 and these were followed by the Chinchillas in 1913 and the American Blue in 1917. The Sables, Martens, Lilacs and Rexes are of more recent development.

The origin of the French Silver and the Himalayan is unknown and very little is known concerning the English and the Belgian. It is reasonably certain, however, that the Silver Gray, the oldest of all domestic rabbits, furnished the essential features of the English type, while the characteristic spotting was developed in some way from one of the common spotted breeds, probably the Belgian *Land Kaninchen*. The Belgian Hare was derived from one of the heavier Flemish breeds, but how it acquired its distinctive hare type and reddish brown color are still matters of conjecture. The original assumption of an accidental cross with a wild hare seems untenable. The fact that type and color have been greatly accentuated in the hands of modern breeders shows that selection has played an important part in the sorting out and development of the distinctive characteristics of this breed.

The Dutch, Polish, Tans and Havanas are racially related. The Dutch was derived from the Brabançon, an animal with similar marking but less distinctive type. According to some accounts, Polish rabbits have been produced from several of the small breeds including Dutch, Silver Grays and Himalayans, but the accepted type is evidently Dutch and it is probable that both were derived from a common ancestor and that each has been used in the perfection of the other. The Tans are said to have originated from an accidental cross between a Dutch and a wild rabbit with selective breeding of the progeny from this mating. The Havana had a similar origin from a mating between a mongrel Dutch marked doe and an unknown sire.

Racially, the original Blue Beveren belonged to the giant group of rabbits. The first animals of this type appeared in a litter of St. Nicholas Giants in 1898. From these, both the Blue and White Beverens have been developed.

The American Blue was developed by the deliberate crossing of a number of blue strains and the selection of desirable types which were finally combined and bred toward present standards. Racially, it is a mongrel breed, but has been developed into a fairly distinct type with a leaning toward the giant class.

The so called Lilacs include animals of several origins. They are all dilute brown and all of them are derived from an Havana ancestor and are, therefore, related to the Dutch group on one side. One variety known as the Gouda was obtained by the cross between the Havana and the Blue Beveren; others have been obtained by the crossing of other varieties of blue rabbits with the Havana. The strains thus produced differ according to the origin of the blue parent and the direction of the selective breeding.

The Chinchillas, Sables and Martens form another racial group. The first of these was the Chinchilla which is said to have been produced by the crossing of a

wild gray rabbit with a blue of unnamed breed and with the Himalayan and the subsequent interbreeding of the progeny of these crosses. The Sables were obtained either as a result of continued breeding of Chinchillas carrying Himalayan albinism or by crossing Chinchillas with Himalayans and the breeding of these animals *inter se*. The so called Martens were obtained in like manner by the crossing of Chinchillas or Sables with Black and Tans. Some Sables and Martens have been produced by the use of albinos other than the Himalayan. This was true of one imported strain in our colony.

All of the animals of this group, together with the Himalayan, are genetically members of the albino series; all of them are racially related to the Himalayan and the Martens are related to the Tans.

The Rex was the first of the short haired breeds. Animals of this kind are said to have been noted in the litters of common gray rabbits for many years, but the first effort to breed them as a distinct variety was made in France about 1919 and from these the present Rexes were developed. It would appear that the Rex was of mongrel origin, but stocks of the original Gillet strain purchased by us from an importer showed unmistakable evidence of Belgian and Flemish ancestry with the Belgian character predominating.

*Racial Integrity.*—With respect to racial integrity and homogeneity, little need be said concerning the older breeds. Most of them have been brought to a high state of perfection and their standards have changed very little over a long period of years. They may, therefore, be regarded as distinct races. There are probably strain differences but apparently they are fairly homogeneous in respect to the more distinctive racial characters. This applies to the true Belgian Hare, Dutch, English, Himalayan, Polish, Silver and Tan.

The racial integrity of other breeds listed above is uncertain. They are not only of recent origin but they have been subjected to repeated hybridization in an effort to develop a desired type and to improve certain characters. Still, some of them have been bred in accordance with rigid specifications for a number of years, and whether we regard them as distinct races or merely as breeds, they probably possess some distinguishing characteristics.

The Rex should be considered in a category by itself. There are other genetically distinct forms of short haired rabbits which are not to be confused with the Rex. The character of the coat distinguishes the Rex from other rabbits, but instead of perfecting a single breed, the rex character has been utilized in the creation of "rex" varieties of other breeds, so that at the present time Rex has no racial significance except in so far as the grafting of this coat on to other stocks may be shown to have produced an effect of a given character (3).

Our own stocks of Rex rabbits are open to the same criticism as it was impossible to maintain these animals in pure line from the original foundation stocks. Hybrid matings were made and from these rex coated animals were obtained and bred with as little outcrossing as possible and with a view to maintaining a type approximating that of the original stocks.

*Hybrid and Factor Stocks.*—The hybrid and factor stocks need not be described

here. Reference to the peculiarities of these animals will be made in the discussion of results.

The age, breed and sex distribution of the population are given in Table I. The population of the colony was divided into two clearly defined age groups, the one composed of adults, the youngest of which was born during the previous spring and were, therefore, about 6 months old, and the other of young stock, the oldest of which was 9 weeks old at the beginning of the outbreak.

Other information concerning the housing and care of the colony has been given in previous papers.

## RESULTS

### *Infection and Morbidity*

Observations on the facility with which infection occurred in different groups of animals and the variations in the severity of symptoms could not be systematically recorded during the epidemic. It was noted, however, that these varied with age, sex, breed and physiological status. For example, no case of infection was found before the 5th day of life and, in several instances, direct exposure during the first 2 weeks did not result in the immediate development of disease. In one instance, the litter of a Belgian doe that had died of the disease was fostered with a doe nursing young of comparable age, and although the fostered young died shortly and at autopsy showed typical signs of the disease, the foster mother's own young remained in good health and were among the last in the colony to develop the infection. It is possible, however, that the apparent resistance shown by some animals resulted from nursing an immune doe and bore no other relationship to age. Animals between 3 and 9 weeks of age, on the other hand, were particularly susceptible and infection spread rapidly through cages containing litters of this age group.

Adults were generally more resistant but occasionally, a lactating doe showed advanced signs of the disease before the earliest lesions could be detected in its litter. Pregnant does were also easily infected. Adult males were most resistant and generally withstood contagion longest.

There were also marked variations in the severity of the disease. In general, symptoms were most severe in young and debilitated animals and the disease ran a rapid course to a fatal termination. Does under the strain of pregnancy or lactation were also seriously ill, but if

relieved, usually showed rapid improvement. All breeds were not equally affected but showed pronounced individual differences in the frequency and severity of constitutional symptoms and, in most instances, the mortality rates varied accordingly. In certain breeds as the Himalayans, adult animals rarely showed constitutional symptoms and no deaths occurred while in others, as the Belgian, infection was followed by rapid collapse and death. Among the Havanas, however, no such correlation was found. The breed, as a whole, was one of the most seriously affected, but the mortality rate was not commensurate, many animals recovering from a grade of illness measured by the extent and destructiveness of lesions on the one hand, and the consequent debility on the other, which would have been fatal in other breeds.

### *Gross Mortality*

The immediate reaction to the disease as indicated by the ease with which infection occurred and the severity of symptoms was in conformity with the ultimate outcome in all groups of animals with the exception noted above. In this case, however, as in the others, the outcome of infection is a more accurate gauge of the reaction of the animals and may be used as an index in judging the importance of various constitutional factors in the determination of susceptibility or resistance.

Mortality rates are summarized in Table I. Animals that died of causes other than pox or were killed for various reasons during the epidemic period are omitted and were not considered in computing mortality rates. Of the 1313 exposed animals, 610 died of rabbit pox, giving a gross mortality of 46.4 per cent.

### *Age*

There were wide variations in the mortality rates of animals of different age groups which corresponded with the clinically observed variations in morbidity. The mortality among animals less than 14 weeks old was 71.8 per cent and 14.7 per cent for those ranging from 7 months to 4 years. The relationship of the adult and young stock mortality rates throughout the epidemic is shown in Text-fig. 1. The first deaths occurred in adults and, for a short time, outnumbered



those in young animals. The number of deaths in young stock increased rapidly with spread of the infection and the mortality curve agrees in general trends with that of the total population. The adult mortality rate, however, after a slow rise continued at a fairly uniform low level, gradually declined and ceased a full week before the last death occurred in the young stock.

TABLE I

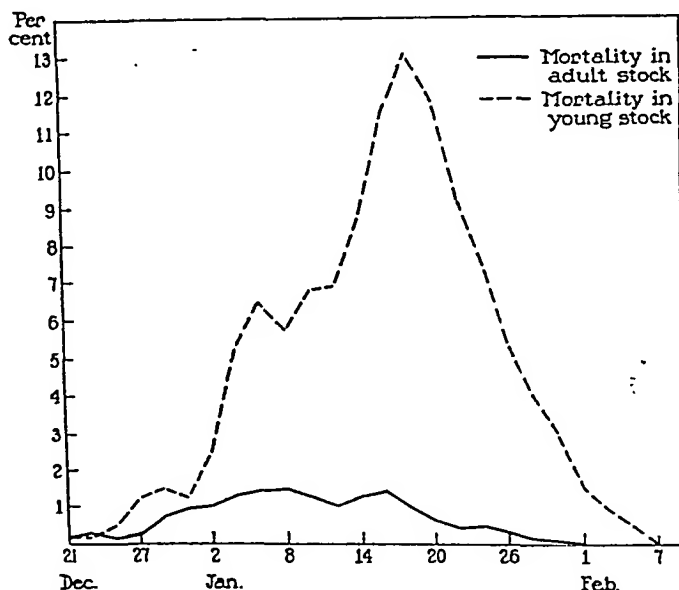
*Distribution of the Population and the Mortality Rates of Various Groups*

| Breed              | Adults         |         |               |         | Young          |         |               |         | Mortality |          |
|--------------------|----------------|---------|---------------|---------|----------------|---------|---------------|---------|-----------|----------|
|                    | No. of animals |         | No. of deaths |         | No. of animals |         | No. of deaths |         | Adults    | Young    |
|                    | Male           | Fe-male | Male          | Fe-male | Male           | Fe-male | Male          | Fe-male |           |          |
|                    |                |         |               |         |                |         |               |         | per cent  | per cent |
| American Blue..... | 1              | 3       | 1             | 1       | 4              | 3       | 2             | 2       | 50.0      | 57.1     |
| Belgian Hare.....  | 10             | 20      | 4             | 6       | 18             | 17      | 17            | 16      | 33.3      | 94.3     |
| Blue Beveren.....  | 8              | 20      | 1             | 4       | 16             | 10      | 10            | 7       | 17.8      | 65.4     |
| Chinchilla.....    | 5              | 8       | 2             | 1       | 4              | 3       | 3             | 2       | 23.0      | 71.4     |
| Dutch.....         | 12             | 17      | 0             | 7       | 18             | 15      | 14            | 14      | 24.1      | 84.8     |
| English.....       | 11             | 30      | 0             | 2       | 3              | 3       | 2             | 2       | 4.8       | 66.6     |
| Himalayan.....     | 9              | 16      | 0             | 0       | 12             | 4       | 9             | 2       | 0         | 68.7     |
| Havana.....        | 12             | 32      | 2             | 6       | 21             | 23      | 18            | 18      | 18.1      | 81.8     |
| Lilac.....         | 2              | 2       | 0             | 0       | 3              | 1       | 0             | 0       | 0         | 0        |
| Marten.....        | 3              | 8       | 0             | 0       | 1              | 2       | 1             | 2       | 0         | 100.0    |
| Polish.....        | 5              | 8       | 1             | 2       | 4              | 10      | 2             | 6       | 23.0      | 57.1     |
| Rex.....           | 11             | 16      | 3             | 5       | 6              | 12      | 4             | 9       | 29.5      | 72.2     |
| Sable.....         | 7              | 14      | 0             | 2       | 4              | 5       | 2             | 2       | 9.5       | 44.4     |
| French Silver..... | 6              | 6       | 3             | 0       | 0              | 0       | 0             | 0       | 25.0      | 0        |
| Tan.....           | 8              | 6       | 2             | 1       | 0              | 0       | 0             | 0       | 21.4      | 0        |
| Hybrids.....       | 98             | 170     | 9             | 21      | 0              | 0       | 0             | 0       | 11.2      | 0        |
| Total.....         | 208            | 376     | 28            | 58      | 350            | 379     | 257           | 267     | 14.7      | 71.8     |

The mortality rate also varied in different age week groups of young stock as is shown in Table II. No deaths occurred before the 5th day of life and among animals 1 week old the mortality was low. The rate increased rapidly with age up to the 4th week and was highest among animals 4 to 9 weeks old. Thereafter, it fell with equal rapidity and reached a low point in animals of 13 weeks. No deaths occurred in animals 14 weeks old, an age which was not represented until near the close of the epidemic.

The mortality among animals in the various age groups showed considerable

variation at different periods of the epidemic, but generally increased during the course of the epidemic and, in most cases, reached a maximum during the 5th week. During the 1st epidemic week, the colony contained young stock of all ages from 1 day to 9 weeks, and deaths occurred in all age groups except those of the 1st and 2nd weeks with a maximum mortality per cent in animals 9 weeks



TEXT-FIG. 1. Comparison of young and adult stock mortality.

TABLE II  
*Mortality Per Cent in Age Groups of Young Stock*

|                         | Days |      |       |       |       |       |       |       |       |       |       |       |       |
|-------------------------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|                         | 1-7  | 8-14 | 15-21 | 22-28 | 29-35 | 36-42 | 43-49 | 50-56 | 57-63 | 64-70 | 71-77 | 78-84 | 85-91 |
| No. of animals.....     | 469  | 526  | 569   | 531   | 566   | 684   | 655   | 574   | 440   | 343   | 275   | 209   | 139   |
| Pox deaths.....         | 3    | 27   | 53    | 66    | 69    | 67    | 91    | 60    | 44    | 27    | 12    | 4     | 1     |
| Mortality, per cent.... | 0.63 | 5.1  | 9.3   | 12.4  | 12.2  | 9.8   | 13.9  | 10.5  | 10.0  | 7.9   | 4.4   | 1.9   | 0.72  |

old. Litters continued to be born until the end of the 4th epidemic week, and although every week up to the 5th contained representatives of the 1 week age group, the only deaths occurred during the 3rd epidemic week. Among animals more than 1 week old, there were deaths representative of every age period during the 2nd, 3rd, and 4th epidemic weeks and in the 5th week there were deaths in all

age periods except in the group 13 weeks old which was present for the first time. The highest mortality rate during the 2nd, 3rd and 5th weeks occurred in animals 7 weeks old and during the 4th week in animals 4 weeks old. In the 6th epidemic week there were no deaths in animals under 5 or over 12 weeks of age and the mortality was highest in the 8 weeks old group. Only three deaths occurred in the final epidemic week, one each in the 7, 11 and 13 week age groups.

It is important to note in this connection that the active period of infection in young animals was rarely longer than 3 or 4 days; in the great majority of cases, infection and death occurred in the same week of age, so that the mortality rate of an age week group is representative of the susceptibility of animals of that age.

Thus, while the most pronounced differences in susceptibility occurred between young and adult stock, there were also marked variations between the different age groups of young animals, and especial significance is attached to the fact that the most susceptible age varied with the epidemic phase, and an age group comparatively resistant during one stage was more susceptible during another and *vice versa*. In general, a fatal termination was most common when the infection was contracted during the 7th week of life, but during the primary and terminal stages of the epidemic, an older group proved to be more susceptible while during the 4th week when the death rate of the entire population was greatest, the highest susceptibility occurred in much younger animals or among animals which at the beginning of the epidemic and during the terminal stages were comparatively resistant.

### Sex

There were 208 males and 376 females among the adult animals that contracted the disease and the mortality rates of these groups were 13.4 per cent and 15.4 per cent respectively. An analysis of the female population showed, however, that a large number were pregnant or lactating during the epidemic, and this apparently contributed to their higher susceptibility, for a comparison of the mortality rates of resting animals of both sexes resulted in no significant difference.

There were pregnant, lactating and resting females in the colony. As a rule, the infection of a pregnant doe resulted in abortion. In other cases, lactation followed pregnancy and eventually, with destruction or weaning of the litter, the doe returned to a resting condition. In many instances, death occurred during the transition from one of these states to another, and it was not possible to determine accurately the effects of each on the final outcome of infection. However, an indication of the influence of physiological status on susceptibility may be obtained by a comparison of mortality rates for females which, because of the duration of pregnancy, lactation or rest could be considered as having acquired

the reactions of these various states. Thus, it was found that there was no difference between the mortality rates of resting and pregnant does, the usual sequence among pregnant does having been abortion, improvement and recovery, but that an increased liability to fatal termination was associated with lactation, the mortality rate of lactating animals being increased to 16.5 per cent and probably would have been higher if lactation had not been terminated in many instances by weaning or death of the litter.

Among the young animals of the colony, the mortality varied from 70.4 per cent in females to 73.4 per cent in males, but inasmuch as the majority of rabbits do not reach sexual maturity until near the end of the 4th month of life, or even later, this difference probably resulted from age period variations or other factors rather than the influence of sex.

### *Breed*

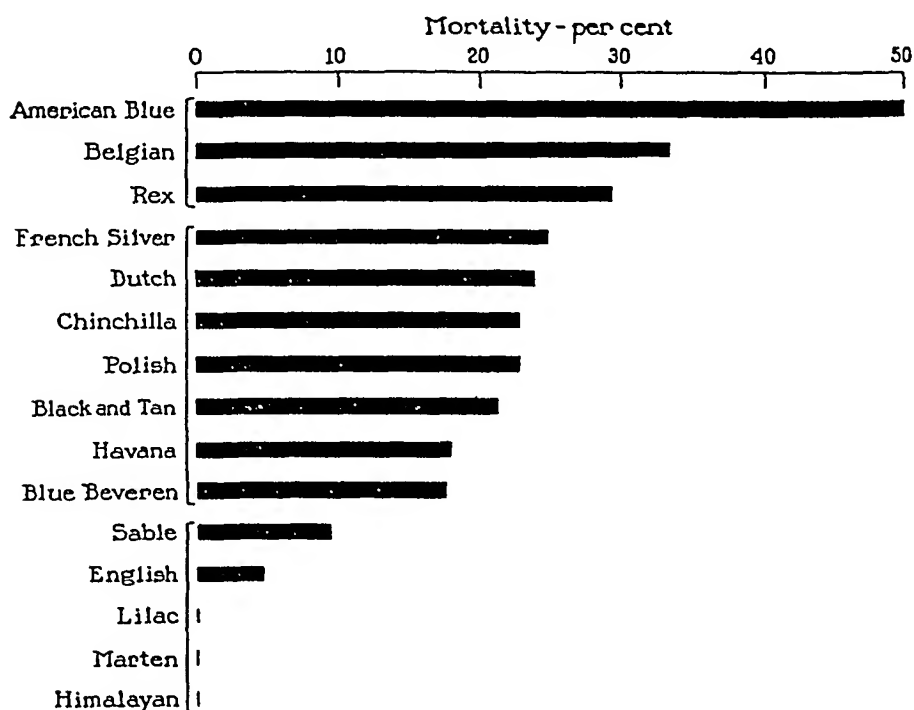
*Pure Breeds.*—During the course of the epidemic, it was observed that animals of different breed showed wide variations in the character and severity of their reaction to infection. This was substantiated by an analysis of the mortality of pure bred stocks and of hybrids, but the position of the various breeds in a mortality scale differed depending on the age group used for comparison. For this reason, young and adult animals were considered separately in Table I which shows the number in each breed and the respective mortality rates.

The susceptibility relations of the various breeds are shown graphically in Text-fig. 2. The significance of the relative position of the American Blues and Lilacs is uncertain inasmuch as these breeds contained only four animals each, but all other breeds were sufficiently represented to allow a determination of relative susceptibility or resistance.

Inspection of this graph suggests a three point division of mortality rates. The American Blue, Belgian and Rex form a group in which the mortality was exceedingly high while the Sables, English, Lilacs, Martens and Himalayans showed a correspondingly low mortality. Other breeds were intermediate and showed graded differences from the Silver in which the death rate was only slightly lower than that of the Rex to the Havana and Beveren in which the mortality was distinctly lower but was still much higher than that of the lowest group.

In comparing mortality rates, it is important to note that in adults

the distribution of the lesions characteristic of the disease varied in different breeds. Among the most susceptible breeds, as the Belgian, cutaneous lesions were comparatively infrequent while visceral lesions were widespread. On the other hand, the resistant breeds generally showed a more extensive cutaneous involvement with comparatively few visceral lesions. In the Himalayan and other breeds in which no deaths occurred, cutaneous papules were atypical, small and usually could be found only after a most extensive search, while visceral



TEXT-FIG. 2. Breed mortality.

lesions were rare even in animals killed at the height of the disease. The Havanas as a whole showed a more or less equal cutaneous and visceral distribution; the visceral lesions were less marked than in the more susceptible breeds, so that animals with severe constitutional symptoms frequently recovered and the death rate was intermediate. In general, a widespread occurrence of visceral lesions resulted in an acutely fatal termination while a cutaneous distribution or localization was associated with a mild or protracted course of disease fol-

lowed by recovery, and the susceptibility of the different breeds varied with the proportion of cutaneous and visceral involvement.

Although a number of breeds occupied comparable positions in the young stock mortality, in most instances the susceptibility relations of young and adult animals were entirely dissimilar. The French Silver and Black and Tans contained no young stock, but a comparison of the mortality rates of the breeds that were adequately represented indicated that the factors responsible for breed variation in adult animals either had not reached full expression in the young stock or were dominated by age factors.

*Hybrids.*—The adult hybrid stock consisted of 268 animals and was divisible into two main groups. One of these, designated as inbred albinos, was a mongrel stock of uncertain composition but shown by breeding tests to have been derived in part from a Dutch-Polish cross. These animals had been closely inbred since 1918. The second group of hybrids was composed mainly of first and second generation crosses from pure bred parents, but also contained a number of animals derived from the inbred albino group by crossing with pure bred stocks. The mortality rate of the combined adult, hybrid population was 11.2 per cent which was significantly lower than the mortality among pure bred animals of corresponding age. The inbred albino family showed a slightly higher rate of 15 per cent while the group of animals derived from matings of these with pure breeds showed a much reduced rate with variations within the group corresponding to the susceptibility relations of the breed used. The only adult  $F_2$  representatives of these outcrosses present in the colony during the epidemic were the progeny of the Albino-Himalayan matings and the mortality of this group (24.3 per cent) was considerably higher than that of the  $F_1$  animals. For the most part, however, these animals represented a group which had been selected or bred for the study of certain physical and functional disorders which they were known to transmit.

The first hybrid generation from pure bred parents numbered 133 adult animals and showed a mortality rate of 6.7 per cent. There were, however, wide variations in susceptibility depending upon the parent breeds. Table III shows a classification of the first generation hybrids in which their pure bred ancestors are considered as relatively

susceptible if the mortality rate of the breed is greater than 17.8 per cent, the average mortality of pure bred animals, and relatively resistant if the rate is less than this figure. The mortality rates of all the various groups thus formed fell into the division designated as resistant, but the group of hybrids obtained from the crossing of susceptible breeds showed a significantly higher death rate than the group resulting from the mating of susceptible with resistant breeds. Moreover, there were no deaths among the animals derived from hybrid matings within the resistant division of breeds.

### *Parent-Progeny Relations*

A further analysis of adult mortality data was made on the basis of a parent-progeny test. For this purpose, breed was disregarded and

TABLE III

*Mortality Rates in Various Groups of Hybrids Based on the Breed Susceptibility of Their Parents*

| Pure breeds  | Hybrid offspring |               |                 |
|--|------------------|---------------|-----------------|
| Matings classified according to susceptibility of parents' breed | No. of animals   | No. of deaths | Mortality       |
|  |                  |               | <i>per cent</i> |
| S × S*.....  | 73               | 7             | 9.5             |
| S × R†.....  | 47               | 2             | 4.2             |
| R × R.....   | 13               | 0             | 0               |

\* S = susceptible.

† R = resistant.

those animals whose parents contracted the disease were divided into three groups according to the outcome of the infection as shown in Table IV. The highest mortality occurred in the group in which both parents died and the lowest in the group in which both recovered.

The group of animals in which both parents died is too small to be of definite significance as a measure of susceptibility to pox, but the difference between the other groups shows clearly a relation between parent and progeny comparable with the relation brought out between hybrids and the pure breeds from which they were derived.

An examination of breeding records showed that the small number of animals in Group 1 was not due to a lack of matings of this class

but to the fact that the progeny of such matings failed to reach maturity and that losses during early life from various causes tended to operate in the same general direction as the observed susceptibility to pox infection.

TABLE IV  
*Relation of Mortality Rates in Adult Progeny and Fate of Parents*

| Fate of parents           | Offspring      |               |                       |
|---------------------------|----------------|---------------|-----------------------|
|                           | No. of animals | No. of deaths | Mortality<br>per cent |
| Both died                 | 4              | 1             | 25.0                  |
| One died<br>One recovered | 37             | 5             | 13.5                  |
| Both recovered            | 110            | 9             | 8.1                   |

TABLE V  
*Relation of Mortality Rates in Adult Progeny to Breed Susceptibility and Fate of Parents*

| Classification of parents        | No. of progeny |                           |                |       | No. of deaths |                           |                |       | Mortality        |                           |                  |                  |
|----------------------------------|----------------|---------------------------|----------------|-------|---------------|---------------------------|----------------|-------|------------------|---------------------------|------------------|------------------|
|                                  | Both died      | One died<br>One recovered | Both recovered | Total | Both died     | One died<br>One recovered | Both recovered | Total | Both died        | One died<br>One recovered | Both recovered   | Total            |
|                                  |                |                           |                |       |               |                           |                |       |                  |                           |                  |                  |
| Both of S breed                  | 4              | 35                        | 47             | 86    | 1             | 5                         | 6              | 12    | 25.0<br>per cent | 14.2<br>per cent          | 12.7<br>per cent | 13.9<br>per cent |
| One of S breed<br>One of R breed | 0              | 2                         | 22             | 24    |               | 0                         | 2              | 2     |                  | 0                         | 9.0              | 8.3              |
| Both of R breed                  | 0              | 0                         | 41             | 41    |               |                           | 1              | 1     |                  |                           | 2.4              | 2.4              |
| Total.....                       | 4              | 37                        | 110            | 151   | 1             | 5                         | 9              | 15    | 25.0             | 13.5                      | 8.1              | 9.9              |

In order to test this relation still further, parents and progeny were classified with respect to the susceptibility of the parent breed as indicated above, and of the parents themselves. The results of this analysis are given in Table V. This table shows first, the fate of



animals when both parents were of a susceptible breed and both died or when one died and the other survived and when both survived; second, corresponding results for animals derived from parents one of which was of a susceptible breed and the other of a resistant breed, and third, the fate of animals derived from parents both of which came from resistant breeds.

In some of the groups formed by this detailed classification, there were no adult progeny in the colony or parents had been discarded previous to the epidemic, while in others representative animals were present in small numbers only. The significance of the reaction to infection shown by some of these groups is doubtful if measured statistically, but the trend of variation is in agreement with results obtained and may be considered as indicative of probable differences that would have occurred had larger groups been available. In brief, the results show that the death rate of adult animals derived from resistant parents of resistant breeds was 2.4 per cent, while the corresponding rate for the progeny of susceptible parents from susceptible breeds was 25.0 per cent.

#### DISCUSSION AND CONCLUSIONS

One of the most striking features of the epidemic of rabbit pox reported in this series of papers was the variation in the severity of the disease shown by animals of different age, breed and physiological status. Other variations in severity were apparently dependent on the epidemic phase in which infection occurred and, as was pointed out in a previous paper, were probably due to changes in the general resistance of the population and in the virulence of the infecting organism.

The mortality rate of 71.8 per cent in young animals as compared with 14.7 per cent in adults gives a clear indication of the importance of physiological processes associated with growth and maturity. The significance of factors of this kind is further emphasized by the variations in susceptibility shown by young animals of different age groups. For example, the highest mortality occurred between the ages of 4 and 9 weeks. During this period, in the course of events, young rabbits begin an independent existence. Weaning, however, is a gradual process, and for a variable period the eating of grain and other food

is supplemented by nursing. In some instances this is continued into the 3rd month, but in the majority of cases weaning is completed by the end of the 8th week. Before the 4th week, when young animals were physiologically adapted to a dependent existence, and after the 9th week when the majority were self-sustaining, the mortality was low. During the period of transition, however, while the profound changes were taking place which would enable an independent existence, and the physiology of the animals was in an unstable or imbalanced state, the mortality was highest.

During the height of the epidemic, the severity of the disease was markedly increased and the infection and disability of does often led to a shortened nursing period and, in other instances, litters were forcibly weaned as a measure of protection for the mother. During this period, the influence of early weaning is indicated by the shift of the most susceptible age from an older group during the early and terminal stages of the epidemic to much younger animals.

These findings are in complete agreement with the well known high mortality among rabbits of this age group from various causes and from gastro-intestinal disorders in particular. It may be concluded, therefore, that the non-specific resistance which is a natural attribute of healthy animals depends in part on a stable and balanced physiological condition. In the imbalanced state of adaptation to an independent existence, resistance is low and susceptibility to disease is correspondingly high. The increased susceptibility to disease at this age is manifest normally by a rise in the death rate from gastro-intestinal infections and in the present instance, this peculiarity of age probably accounted for the increased mortality among animals of this group. This period of increased susceptibility to disease in the rabbit suggests an analogy with the apparent increase in susceptibility to infection among infants which occurs around the 9th or 10th month (4).

Further evidence of the influence of physiological status is seen in the occurrence of severe and disabling infections and a higher mortality among lactating does than in other classes of adult animals.

Sex of itself apparently exercised a minor influence on susceptibility. In young animals there was no clearly defined difference between the sexes, but among adults it appeared that the tax imposed on females

by reproductive activity diminished their chances of survival to an appreciable extent as compared with males and other classes of females.

Breed variations in susceptibility are of especial interest. Among young animals, the factor of age appeared to overshadow all other distinctions but in adults, breed as represented by this population proved to be a factor of the foremost importance.

The question arises as to whether breed differences were referable to racial distinctions or to other peculiarities and relations. Disregarding the American Blue, it is interesting to note that in the high mortality bracket (Text-fig. 2) there are two breeds, the Belgian and the Rex, which in this instance was racially related to the Belgian and so far as is known, was the only relative of the Belgian in the pure bred population. In the middle bracket we find every member of the Dutch group; these include the Dutch, Polish, Tan and Havana, in the order given, which is the approximate order of racial relation. In like manner, the Himalayan group, with the exception of the Chinchilla, is concentrated in the lowest bracket.

Race may, therefore, be regarded as a factor of considerable importance, but such conformity in the behavior of racial groups was hardly to be expected in view of the fact that some breeds might possess conflicting racial tendencies or a lack of homogeneity due to recent crosses which had been made in the process of perfection. But it seems that in some instances, even these crosses may have played a part in determining the scale of relative susceptibility within the group. Thus the Sable and the Marten are racially and genetically more closely related to the Himalayan than is the Chinchilla. The Sable of this stock is the product of a Chinchilla-Himalayan cross and occupies a position intermediate between the two, while the Marten comes from a Tan-Sable cross and goes into a still lower class.

As was pointed out above, the animals of this group are genetically members of the albino series; Sables and Martens are Chinchillas. The distinctive feature in the coat of the Chinchilla proper is produced by the agouti or wild type factor; this factor has been displaced in the Sable with the production of a uniform color while in the Marten, the agouti factor is replaced by factors for Black and Tan pattern. Moreover, the Chinchillas of this stock were homozygous for Chinchilla albinism and for the agouti factor, while the majority of the Sables

and Martens were heterozygous and carried Himalayan albinism as a recessive character. The significance of the agouti factor in the Chinchilla is by no means certain, but it is of interest to note that the only other animals which possessed this factor were the Belgians and the Rexes.

There is a suggestion of a similar relation among animals that were genetically brown as distinguished from the alternative of black. Brown breeds were represented by the Havana and Lilac, but in this particular instance, a brown cross had been introduced at some time to brighten the coat of the Beverens and most of the stock still carried brown as a recessive character. The rating of the Lilac is uncertain, but the only other breeds that carried brown were the Havanas and Beverens and these gave an almost identical result, despite the existence of other marked differences.

While there is very little information concerning the physiological significance of genetic factors affecting the color and character of the coat, it is well to remember that these factors do influence physiological activity in so far as the coat is concerned and that they may affect physiological functions in other ways. For example, the factor for yellow is lethal in mice and there are numerous instances in which peculiarities of function or pathological conditions appear to be associated with particular color genes in other animals. Hence, it may be that particular combinations of color genes exercise an appreciable influence on susceptibility to disease. The peculiarities in the response of certain groups of rabbits, as pointed out above, would suggest this possibility, and factors of this order may play a part in determining racial characteristics.

The question now arises as to why one racial group should be more or less resistant than another. In the present state of knowledge concerning the value of given racial and genetic characters, this question cannot be answered satisfactorily, but some information is available which undoubtedly has a bearing on this problem. This has been obtained mainly by genetic studies aimed in the first instance at a separation of essential racial characters from variations which have been incorporated in the stock by chance association, and a determination of the influence of these characters.

Examination of the results recorded in Text-fig. 2 gives very little

indication as to the cause for such wide variations in susceptibility among pure breeds. There is, however, some evidence to show that, other conditions being equal, the smaller animals and animals of a linear type are more resistant than the larger, more compact types, but clearly this is not the main consideration in the present instance. The larger animals as a class are toward the top, but the Blue Beveren is well down the scale and the smallest of all, the Polish, occupies a mid-position. Again, the English and Himalayan, as representatives of the slender type, are definitely among the most resistant breeds, but the Belgian, the raciest of all, is at the top of the list. It is evident, therefore, that while racial characters of this order may have a value in determining or indicating susceptibility, their influence may be completely overshadowed by other factors.

The analysis of results obtained in hybrids throws some light on this subject. The reaction of the hybrid population as a whole indicated that heterozygosity favored general resistance to the disease. The converse, that homozygosity favored susceptibility, was suggested by the reaction of the Himalayan-Albino hybrids which were relatively resistant in their first generation, but highly susceptible in their second. However, the variation in the susceptibility of different hybrid groups showed that the characteristics of parent breeds were transmitted to the offspring in the manner of definite hereditary factors. Thus, hybrids derived from the crossing of susceptible breeds were found to be more susceptible than those obtained from crossing susceptible and resistant breeds, while the hybrids resulting from the crossing of resistant breeds showed no mortality. The reaction of the group derived from the crossing of susceptible and resistant breeds (Table III) showed, however, that while the breed factors determining the susceptibility of one parent may be inherited as recessive characters, they are not completely dominated by those concerned in determining the reaction of the other parent breed but exert an appreciable influence on the susceptibility of the progeny.

Disregarding breeds and reducing the analysis to the basis of a parent-progeny test, the influence of inheritance was still perceptible. It thus appears that factors other than those characteristic of a breed, or separable from distinctive racial characters, played an important part in determining the susceptibility of adult animals, including pure

bred and hybrid stocks. The nature of these factors is a matter of considerable importance.

As was pointed out above, some pure bred and hybrid stocks were known to carry mutant genes capable of the production of more or less serious disorders. There is evidence for and against the assumption that factors of this class may have contributed largely to the susceptibility of certain groups of animals. An analysis of the Belgian population furnishes an excellent example of the possible influence of genetic constitution on susceptibility to disease.

The Belgian population contained some excellent specimens of the Belgian Hare, but few, if any, animals which genetically could be regarded as normal. The Belgians carried a number of definitely lethal factors which were transmitted from parent to offspring as recessive characters with an appreciable alteration in the functional capacity of heterozygous individuals. The precise number and character of these abnormalities is unknown, but there was one group which affected nutritional capacity with respect to vitamin metabolism; another which was associated with abnormalities of the hematopoietic system; and still others which produced a variety of abnormalities too numerous to mention (5). No group of animals in the colony was known to carry so many detrimental characters. Moreover, the death rate among these animals from all causes, including young and old, was exceedingly high. They were highly susceptible to snuffles and to pneumonia which was the chief cause of death among animals past the weaning age. They had also been found to be susceptible to mouse typhoid in epidemic form and in this instance, pneumonia was a constant finding in fatal cases.

The high mortality from pox was not unexpected and the disease invariably pursued an acute course with the development of pneumonia in fatal cases. With the exception of two females, the survivors were animals which were not in the direct line of transmission of the lethal factors referred to and neither of these carried the complete series of lethal factors. The survivors were mainly heavy weight animals of uncertain lineage. That is, every pure bred animal in the colony that was a carrier of the two principal lethal factors died of pox; one hybrid female of this type survived. One male of this line, which was not in the colony at the time, escaped exposure and infection as was shown by a subsequent test of immunity.

This might appear to be a clear case of unusually high susceptibility dependent upon the concentration of unfavorable genetic factors. But, it is not certain that this was the case as the disease pursued essentially the same course in animals which, so far as tests had gone, were not transmitters of this particular group of lethal factors; they were, however, transmitters of other functional disorders. What these animals possessed in common with others of the group is not known, but it appears that these characters, together with known genetic factors contributed largely to the reaction of this breed to pox infection.

The Rex, Dutch and Polish were also carriers of factors, characterized by disturbances of endocrine function, which produced lethal effects in young animals and caused more or less disturbance of physiological processes in heterozygous adults (6, 7). Among the Dutch and Polish stocks, however, there were striking examples of animals known to be transmitters of the lethal genes and to have developed the characteristic symptoms of the hereditary affection in early life, which not only survived the epidemic, but developed unusually mild infections. This was not true of the Rex population which reacted in much the same way as the Belgians.

There is little definite information concerning the Silver members of the population. Along with the Belgians and the Rex, they were the most difficult animals in the colony to raise and maintain in good physical condition. They were highly susceptible to snuffles and other infection; they were transmitters of several physical abnormalities and the litters contained a large proportion of poorly developed and non-viable young concerning which no definite information has been obtained. The stock might be described, therefore, as containing degenerate characters of unknown nature. These may have operated to lower the resistance to pox infection as in the case of the Belgians and Rex.

Other carriers of unfavorable genetic factors were the Beverens, English and Martens. The affections known to be transmitted by these breeds were highly fatal in young animals; adult Beverens transmitting these characters were also affected at times, but in the English and Martens the disturbance of function appeared to be limited to young animals. There were deaths among the Beverens from gastrointestinal disorders during the pre-epidemic period and during the

early stages of the epidemic, and these may have eliminated the most susceptible members of the population, but barring an assumption of this kind, there is no evidence that the mortality from pox among the Beverens was affected at all by genetic factors of the kind mentioned and there was certainly no effect on the English and Martens.

Other breeds were not known to be transmitters of serious functional disorders. Several of them were transmitters of physical abnormalities, some of which were more or less disabling but did not appear to affect the vitality of the stock in other respects.

From what has been said concerning heritable characters, including those of known and unknown nature, it will be seen that most of the so called factor stocks, or transmitters of serious functional disorders, are in the upper brackets of the susceptibility rating (Text-fig. 2); analysis of hybrid susceptibility showed that the closest relation existed between parent and progeny and that the characters which affected susceptibility were inherited subject to modification through the action of other influences.

In general, therefore, the evidence obtained indicates that in the adult population, susceptibility of groups and individuals to pox infection was a function of racial factors on the one hand, and of genetic factors separable from those characteristic of given races on the other. Factors which are detrimental in themselves usually tended to diminish resistance, but this was not invariably the case. If their action is such that the detrimental effects are limited to a given period of life, it appears that the subsequent effect of such factors may be favorable rather than unfavorable. Factors of the two groups are inherited independently and the final expression of susceptibility or resistance is a result of their interaction.

In the previous paper of this series, it was shown that the occurrence of the epidemic was intimately related to the action of environmental factors which produced a profound disturbance in the health and functional efficiency of the population during the period preceding the epidemic outbreak. We now find that the severity of the epidemic was definitely influenced by the composition of the population with respect to such constitutional factors as age and race and the distribution of genetic factors of various kinds. Factors which tend to produce lethal effects when pure or to produce serious disturbances of



function in homozygous individuals with an appreciable effect on the functional efficiency of transmitters, were usually associated with low resistance to pox infection in pure bred and hybrid stocks. Factors of this order undoubtedly contributed to the production of the differences in susceptibility displayed by different racial groups and may have been largely responsible for these differences. But, there is some evidence to show that other factors, such as those which determine the color and character of the coat, may also exercise an influence on physiological processes and thus play a part in the reaction to infection. Hence, while it has been definitely established that susceptibility to pox infection in adults is a function of racial characters on the one hand, and of factors separable from the racial complex on the other, the relative value of the two groups of factors, individually and collectively, cannot be estimated until they have been separated and tested under appropriate experimental conditions. Moreover, it is not to be inferred that susceptibility to infection as presented in this instance implies a common or pan-susceptibility to infection or disease of all kinds. This is definitely not the case as has been shown by experiments carried out in this laboratory, using some of the same racial groups upon which this study is based (8, 9).

In conclusion, it may be said that the results obtained from the study of the epidemic of rabbit pox reported in this and previous papers furnish a striking example of the epidemiological importance of factors affecting the population, other than those concerned with the presence of an infecting organism and the specific immunity of the population. The importance of these two elements is well recognized, but on the basis of the present investigation, the factors to be emphasized are first, environmental factors which affected the population as a whole and second, constitutional factors which rendered individuals more or less responsive to the action of environment. Age proved to be a factor of the foremost importance, sex was of less significance, and in the adult population, race and constitutional factors associated with particular racial groups but inherited independently, exercised a profound influence on susceptibility. In the final analysis, therefore, the outcome of infection and the character of the epidemic may be regarded as functions of constitutional factors acting under the influence of environment.

## SUMMARY

The epidemiological significance of age, race, sex, genetic constitution and physiological status were studied by means of a differential analysis of the mortality data derived from a devastating epidemic of rabbit pox and, with the exception of sex, were found to be factors of the utmost importance in the determination of susceptibility.

Young animals were more susceptible than adults and although the most susceptible age varied with the epidemic phase, it corresponded in general with the period of weaning. The influence of physiological status was further indicated by the increased susceptibility incident to lactation.

Racial variations in susceptibility were obscured by age factors in young animals, but were of profound importance in the adult population and formed the most significant feature of the analysis. A high degree of conformity was found in the susceptibility of racially related breeds, and this similarity in behavior increased with the proximity of relationship. Moreover, a study of the hybrids obtained from the crossing of pure breeds showed that two separable groups of hereditary factors were concerned in the determination of breed susceptibility, one group consisting of essential racial characters, the other of constitutional factors incorporated in the stock by chance association, and that the final expression of susceptibility or resistance was the result of their combined interaction.

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# RABBIT POX

## REPORT OF AN EPIDEMIC

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### PLATE 14

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Within the period of  $2\frac{1}{2}$  years, the rabbit breeding colony maintained at the Institute for studies on constitution has been the locus of three separate epidemics of a disease, which because of its similarity to small pox in man, has been called rabbit pox. The first epidemic occurred in the spring of 1930 and was limited to a small colony of animals housed in a single room. The disease was characteristically mild with low morbidity and mortality rates. As soon as its contagious nature was recognized, animals presenting the clinical features were segregated or sacrificed, with the resulting abatement and disappearance of the disease in epidemic form. A few transmission experiments were attempted but without definite success. This outbreak was not studied systematically, but aerobic and anaerobic cultures of blood and tissues from animals with active lesions and animals that died of the disease were negative.

The colony was thereafter maintained under vigilant scrutiny for the first evidence of a recurrence of the affection, but no case was detected until the latter part of 1932, when the disease again appeared, this time with explosive violence and in a highly malignant form. The clinical, pathological and epidemiological aspects of this second epidemic have been the subjects of reports by Greene (1-5). Transmission experiments conducted by Pearce, Rosahn and Hu (6) were successful, and the etiological agent was identified through clinical, pathological, immunological and host-range experiments as a filterable virus, qualitatively related to vaccine virus, but more virulent.

With the subsidence of the second epidemic approximately 2 months

after its onset, the disease was absent from the colony as far as clinical manifestations were concerned, until December, 1933, exactly 1 year after its second appearance, when a third outbreak occurred in an isolated animal room separated by corridors and stairways from the main breeding rooms of the colony. Experience with the pox during the second epidemic had demonstrated its devastating effect, as indicated by a mortality rate of 610 animals or 46.4 per cent of the total population, and a morbidity rate of more than 90 per cent. With this background, and in order to avoid loss immediate measures were instituted with the object of delimiting the disease to the room in which it was first noted. These efforts first took the form of strict isolation and the sacrifice of animals presenting severe clinical signs of disease. Subsequently, wholesale vaccination of every individual in the colony was undertaken, employing dermovaccine virus as the inoculum (7). This procedure was based on the observation made during the epidemic of 1932-33 that vaccine virus immune animals possessed some degree of immunity against experimental rabbit pox. The present report is concerned with the third and most recent epidemic of rabbit pox. In this instance, the chief interest arises from the opportunity afforded for a comparison of two epidemics in a population derived from a common ancestry, and for a comparison of the behavior of presumptive immune with normal susceptible individuals.

### *Materials and Methods*

For convenience, the epidemics of 1930, of 1932-33 and of 1933-34, have been designated first, second and third epidemics respectively. The observations made during the first epidemic have not been reported. The second epidemic was studied in its spontaneous aspects by Greene, and from the standpoint of etiology and transmission by Pearce, Rosahn and Hu.

The room in which the disease was noted in December, 1933, is on the third floor of a building which is used mainly for animal quarters and is at the opposite end of the floor from the room designated as D in Greene's report (4). The main breeding colony is on the first floor of a different building. The room contained approximately 200 rabbits, housed in individual cages on racks suspended from the ceiling in parallel rows from east to west. In addition there were 110 mice in small boxes located in the northern end.

The rabbit population was heterogeneous both as regards racial and familial attributes and as regards their use for breeding or experimental purposes. At the onset of the epidemic the room housed 173 rabbits. 49 of these, 18 bucks and

31 does, were breeding stock, including standard bred and hybrid animals falling into three general categories: a few were known to transmit characters not at the time under investigation; others were being held for test purposes; and still others were being held as reserves. The experimental group comprised 124 standard bred and hybrid bucks which were distributed according to the type of investigation pursued as follows: experimental syphilis, 78; experimental yaws, 15; the Brown-Pearce tumor, 31. In addition, there were three litters of nursing young born in the room before the epidemic nature of the infection was recognized, one litter of nine born in the room on Dec. 18 during the height of the epidemic, and two litters of five and five which were transferred to the room on Dec. 18 from the main quarters of the colony for reasons which will be described below. The experimental animals were located on racks in the southern part while the breeding stock was segregated in the northern section. Between the two groups was an intervening empty rack. The room was well ventilated and adequately illuminated, and the diet comprised a mixture of grains and grain products with alfalfa, mineral salts and a molasses binder. This was supplemented with hay and a free supply of water.

The study of the disease was limited to the animals which developed spontaneous infections. Transmission and immunity experiments were contemplated but not pursued because of the danger that dissemination of the disease might be given an impetus through the introduction of material known from previous experience to be highly contagious. Identification of the causative agent was thus dependent on clinical and pathological evidence in conjunction with the observed reaction of animals known to be immune or susceptible to the virus of rabbit pox responsible for the second epidemic. Frequent careful clinical examinations of all animals were made, and the presence or absence of any signs of infection were recorded. Animals which died or were sacrificed were examined post mortem, and representative tissues were fixed in formalin and stained with hematoxylin and eosin.

The  $\chi^2$  test of homogeneity which has been employed for the statistical analysis is described by Fisher (8), who also gives tables for translating various levels of  $\chi^2$  into terms of probability. Significance has been ascribed to values of  $P \leq 0.01$ , that is, a difference is considered to be significant when the chances of its occurrence by random sampling alone are 1 or less than 1 in 100.

### *Onset and Course of the Epidemic*

*Adults.*—It will be noted that a majority of the experimental stock had been inoculated with *Treponema pallidum*. One large group of animals was under investigation in experiments designed to alter the reaction of the rabbit to experimental syphilis, especially in an attempt to produce a generalized macular eruption similar to that seen in the secondary stage of human syphilis. For this purpose the sides and flanks of the test animals, together with a group of controls had been clipped shortly after intratesticular inoculation with syphilitic virus

on Nov. 1, 1933. These animals were examined daily for any evidence of a macular syphilid eruption.

After inoculation the orderly progression of the usual phenomena of syphilitic infection was observed. On Dec. 7, 5 weeks after intratesticular inoculation, a diffuse macular eruption was noted on the ears of one test animal, which at the time had a rectal temperature of 105°F. The eruption was easily visible in ordinary daylight, but was most evident on transillumination. When first observed, this macular rash was interpreted as representing the syphilis eruption which was the object of the experimental procedure. Subsequent events showed that this interpretation was incorrect.

On Dec. 8 the macular eruption had become more intense, and on the following day there were several discrete elevated pink and red papules easily discernible on both ears and on the trunk. On Dec. 9 four additional animals were observed with many small discrete papules on the clipped areas of the sides, and a fifth, which had appeared bright and alert the day before, was found dead. At autopsy there were present in the organs and tissues lesions indistinguishable from those noted during the second epidemic. On Dec. 11 three additional animals were found dead with characteristic lesions, and three new clinical cases were observed. 1 day later nine fresh cases were noted. Thus, within 5 days after the first recognition of a macular eruption in a test animal, lesions indistinguishable from those of rabbit pox were seen in 22 animals, four of which died. Of particular note was the fact that the pox lesions were observed in animals inoculated with *Tr. pallidum* and also in control uninoculated animals.

The majority of the animals in the room were under observation with respect to their reaction to different inoculated diseases. In order to preserve as many of these experimental animals as possible, and because of the danger that the disease might spread it was decided to interfere. A strict quarantine had been imposed as soon as the pox nature of the disease was recognized. In addition, every severely affected animal, a total of 18, was sacrificed on Dec. 12.

This procedure appeared to check the incidence of new cases, yet fresh infections were recorded at frequent intervals. As noted above, the first active case was observed on Dec. 7, and the first death 2 days later. The last new case was observed on Jan. 6, 1934, 31 days after the first clinical case was recognized, and the last death on Jan. 9. 58 positive instances of infection were seen in a population of 173 adult animals, a morbidity of 33.5 per cent. These cases were distributed according to the time of their recognition as follows: Dec. 7, 1; Dec. 8-14, 28; Dec. 15-21, 18; Dec. 22-28, 10; Dec. 29-Jan. 4, 0; Jan. 6, 1. 29 or 50 per cent of the total number of infections were recognized in the first 8 days of the epidemic while 47 or 81 per cent were noted in the first 15 days. The distribution frequency of new cases thus has a distinct skew to the left, significantly more cases having occurred in the first half of the epidemic than in the last ( $\chi^2 = 44.8$ ,  $P < 0.01$ ).

The incidence of spontaneous deaths among the adult stock was obscured by the fact that 19 infected animals were sacrificed early in the epidemic. In

spite of this drastic procedure, seven animals died in the interval between Dec. 9 and 22, and four more expired between Dec. 26 and Jan. 9. A prominent feature was that early in the epidemic deaths usually occurred within 2 to 4 days after the first appearance of clinical signs, whereas in the latter half clinical signs anteceded death by a longer interval of 1 to 2 weeks.

*Nursing Young.*—On Dec. 7, at the onset of the epidemic, the room housed three litters aged 5, 17 and 23 days respectively. All survived the epidemic period without any clinical signs of infection. A fourth litter of nine was born in the room on Dec. 18 at the height of the epidemic. Careful clinical examinations never disclosed any manifestations which could be ascribed to the pox. Seven of these survived the epidemic, the others having died at birth or shortly thereafter. The fact that this group escaped infection was further evidenced by a positive reaction in three of six animals later vaccinated with dermovaccine virus. Previous work had already demonstrated that recovered pox animals possessed a high level of immunity against vaccinia, and were refractory to inoculation with dermovaccine virus.

The pox was confined to the room in which it was first noted until Dec. 18 when its dissemination to the main animal colony was evidenced by typical symptoms in a 48 day old rabbit, one of a litter of five. This entire litter and the doe were immediately transferred to the epidemic room. On the following day the infected animal was found dead, and at autopsy the characteristic lesions of rabbit pox observed during the 1932-33 epidemic were noted. After transfer the remaining four were symptom-free for a short time. On Jan. 1, 1934, a second animal in the litter was found dead with characteristic pox lesions; on this date also the other three all had clinical evidences of infection and were sacrificed.

A second litter comprising five rabbits 48 days old, together with their mother, was also transferred to the epidemic room on Dec. 18 because of their proximity to the first infected animal in the main colony. On Jan. 1 one animal of this group was found dead with characteristic pox lesions, and all four of the others showed clinical signs of infection. Two of these were discarded on Jan. 12, at which time they were apparently recovering, while the other two recovered. The significance of the observation that these two litters contracted the disease while the four litters originally in the room did not, will be discussed below.

### *Incubation Period*

Ordinarily, the animals in the room in which the disease was first noted were under the sole charge of one caretaker who came in contact with no other animals. But on Nov. 26, 11 days before the first case of pox was noted, another man was assigned to Sunday duty in this room. Investigation showed that this man was the caretaker of animals from laboratories in which vaccine virus was being used, and that several deaths had occurred rapidly among the animals inoculated with this virus. It was also revealed that he had fed and watered his own animals before coming to our room. It is highly probable that this man acted as a carrier



of the virus, and the incubation period in this instance can thus be placed fairly definitely at 11 days.

The two litters which were transferred to the epidemic room belonged to a small group under investigation by the same persons who were conducting the experiments on the animals in which the pox first appeared. Until Dec. 9, when the third floor animal room was placed under quarantine, the animals quartered therein and these two litters in the main animal quarters had been handled almost daily by both persons. Since the first case of pox in the third floor room was noted on Dec. 7, and the first instance of infection in the main breeding room was observed in one animal of these two litters on Dec. 18, the incubation period in this instance was at least 9 to 11 days. These two estimates, 11 days in the first case and at least 9 to 11 days in the second, are comparable to the incubation period noted by Greene in the previous epidemic.

### *Clinical Manifestations*

The clinical manifestations were with minor exceptions identical with those observed during the second epidemic by Greene. Affected animals were usually listless and apathetic, refused food and frequently became rapidly prostrated. Temperature records of 105°F. were not infrequent. Among local clinical signs the most striking were skin and mucous membrane lesions, which were noted in all cases.

The first skin lesion was usually a macular eruption on the ears or sides. Within 24 hours, papules made their appearance, and usually within 36 hours the papular eruption had become generalized. Papules were most frequently noted over the shaved areas, but careful examination revealed their presence also in fur covered skin. These lesions were usually discrete but sometimes coalescent, and as a rule appeared to be superficial. In general, the skin lesions differed from those noted in the second epidemic in two respects: They tended to be small, rarely attaining a size larger than 2 mm. in diameter, and hemorrhagic papules occurred with far greater frequency. All stages in the development from macules to papules were usually present, but pustules were not observed. Healing occurred with the formation of a dry crust which usually became covered with fine silvery scales and was soon desquamated. The complete absence of scars following the disappearance of skin lesions was noteworthy.

In addition to the papular lesions in the skin, papules were also noted with great regularity in the mucous membranes of the lips, gums, nares, conjunctiva and occasionally, around the anus and genitalia. Mucous membrane lesions were usually discrete and umbilicated.

The next most frequent organ involved clinically was the eye. Eye lesions, as a rule, developed early in the course of the disease as a unilateral or bilateral blepharitis, characterized by a thick tenacious discharge, eversion of the lids, particularly the lower, and a matting of the eyelashes in the secretions. The blepharitis in many instances was accompanied or followed by keratitis but the iris was never involved.

Two other clinical manifestations were encountered with great regularity. The first of these was generalized lymphadenopathy, particularly apparent on examination of the popliteal lymph nodes, which were consistently enlarged, indurated and tense. Inguinal and axillary lymph nodes were also usually palpable. The other was an orchitis in male animals. This was observed less frequently than in the 1932-33 epidemic, and was evidenced in some instances by an edema of the scrotum or testicle, and in others by the formation of small, hard, discrete areas of nodular induration in the testes.

Nasal discharge and diarrhea were of variable occurrence. In the early stages the nasal discharge was usually thick and tenacious. As the condition progressed, the secretion usually became hemorrhagic, at first freely flowing and later followed by the formation of inspissated hemorrhagic crusts which obstructed the nasal passages and caused stertorous respiration. Diarrhea was usually initiated by loose, formed stools which rapidly became liquid in consistence and some times laden with mucus. With the loss of sphincteric control, there was frequently dribbling of urine and fluid feces.

The above gives a composite picture of the disease as it was encountered in the epidemic under discussion. Individual animals, however, varied considerably in the extent and degree of their clinical signs. In very mild cases the only evidence of infection was enlargement of the popliteal lymph nodes which was frequently not sufficient to warrant a positive diagnosis. At the other end of the scale were prostrated animals presenting all of the clinical signs enumerated. In between these extremes were animals with various combinations, both as regards number and degree, of the clinical manifestations described. The extent of involvement was, however, of no certain prognostic import. Animals with severe hemorrhagic papular lesions of the skin were known to recover while others in which the predominant external evidence of the disease was an ophthalmia, were known to die.

### *Pathology*

The autopsy findings in the comparatively small number of animals examined post mortem corresponded closely to the observations during the second epidemic. No attempt will be made to describe in detail the gross and microscopic findings at autopsy. Greene made extensive studies of them during the second epidemic. Only a few characteristic findings will be described to indicate the many points of similarity between the two epidemics.

*Gross Findings.*—The most frequent findings at autopsy were papular lesions of the skin and the mucous membranes of the lips, tongue and conjunctivae. Most of the animals were excellently nourished as indicated by fat. Frequently, the nostrils were partially occluded by thick, hemorrhagic crusts. The pleural cavities usually were not involved, but in two instances a hemothorax was present.

The most frequent finding in the lungs were numerous scattered pearly white indurated nodules superficially located. A few lungs were normal in the gross, but others had scattered bronchopneumonic areas. Focal lesions were not found in the heart although, occasionally, the myocardium was soft and flabby. In some instances the peritoneum was not involved while in others a diffuse fibrinous peritonitis was a prominent feature. Occasionally, small petechiae were noted in the intestines. The liver was usually slightly enlarged, of a yellowish brown color, and frequently showed a scattering of small pearly white nodules on the surface and on cut section. The spleen occasionally presented numerous superficial white nodules of the type found in the liver and lungs, and as a rule was large, soft and swollen. Focal lesions in the kidneys were not seen, but in a few instances these were noted in the adrenal cortex. Marked lymphadenopathy was present in most instances.

*Microscopic Findings.*—The characteristic microscopic lesion consisted of a central area of necrosis surrounded by a zone of small round cells, edema and hemorrhage. In confluent lesions the necrosis predominated. In early cases the lesion was seen to have its inception as a perivascular infiltration of small round cells with associated swelling and distortion of the endothelial lining of small blood vessels and lymphatics.

In the skin, the lesions were not confined to the chorium as in the second epidemic, but were also seen in the epidermis (Fig. 1). In several instances the chorium was surprisingly free of any infiltrative process while in the epidermis, vacuolization, vesicle formation and necrosis were prominent features. In a few instances the lesion had extended through the Malpighian layer and involved the chorium in addition to the epidermis. Only rarely was the chorium alone involved.

#### DISCUSSION

The clinical and pathological similarities between the epidemic disease of 1933-34 reported here and that of the previous year described by Greene leave no doubt that the two infections were caused by closely related etiological agents. The characteristic macular and papular eruption on the skin, the umbilicated papules on the mucous membranes of the lips, nose and gums, and on the tongue, eyes and genitalia, the blepharitis and ophthalmia, the lymphadenopathy and the orchitis, were all essentially the same in the two epidemics. Moreover, the characteristic finding at autopsy of focal and diffuse lesions of the skin and special organs, coupled with a microscopic picture which showed a central area of necrosis surrounded by mononuclear infiltration, edema and hemorrhage, usually intimately related to damaged blood vessels and capillaries, were also common to the two

epidemics. There is, however, certain contributory immunological evidence which taken together with the clinical and pathological similarities makes the close relationship of the etiological agents responsible for the two epidemics fairly conclusive.

At the onset of the second epidemic there were a number of animals which had been in the colony at the time of the first outbreak. The fact that all of these animals developed the disease during the second epidemic indicates either that they had not contracted the affection during the earlier epidemic, or if they had, that the resulting immunity had been lost.

However, by actual test, survivors of the second epidemic were found to be immune to reinoculation with heavy doses of active virus, and this immunity was shown to last for several months. Since there was strong evidence for the belief that every adult in the colony during the second epidemic had contracted the disease, surviving animals presumably possessed a specific immunity. The immune status of a few litters born towards the close of the epidemic was not known with certainty since these never presented any signs of infection, although they were born and raised during the pandemic period. The status of animals born to presumably immune parents mated after the termination of the second epidemic was also conjectural, but because none of these young ever showed any of the disease manifestations, they were all classified as presumably susceptible. Thus with respect to their immune status, there were two general groups and one sub-group of animals in the colony in the period immediately anteceding the third outbreak: presumptive immunes or those that had survived the second epidemic; and presumptive susceptibles, or animals born after the termination of the second epidemic. The sub-group consisted of animals whose status was unknown. These had been born toward the end of the second epidemic but clinically had escaped infection.

Table I presents a classification of the adult animals in the epidemic room according to their presumptive immunity or susceptibility to the causative agent of the second epidemic, and as to the presence or absence of pox lesions in the third epidemic. It is seen that only 2 or 2.2 per cent of 92 presumably immune animals developed the characteristic lesions of the prevailing infection in the third epidemic

while 56 or 69.1 per cent of 81 presumably susceptible animals showed clinical signs of the disease. The difference between the two groups is highly significant statistically ( $\chi^2 = 86.4$ ). Moreover, the disease was not limited to experimental animals as is shown by the fact that 3 or 50 per cent of the presumably susceptible breeding stock rabbits developed lesions as contrasted with 53 or 69.1 per cent of the presumably susceptible experimental animals. The difference between the two groups in this case is not significant ( $\chi^2 = 1.0$ ).

The two animals noted above which were presumably immune to the virus of the second epidemic but which nevertheless contracted the disease during the epidemic of the following year, are of particular significance. These two animals, brother and sister, were born on Jan. 8, 1933, toward the close of the second

TABLE I

*Classification of Adult Animals Exposed to the Epidemic Pox Disease of 1933-34, with Morbidity Rates*

| Group                   | Presumably immune* |                  |          | Presumably susceptible† |                  |          |
|-------------------------|--------------------|------------------|----------|-------------------------|------------------|----------|
|                         | No. of animals     | No. of pox cases | Per cent | No. of animals          | No. of pox cases | Per cent |
| Breeding stock.....     | 43                 | 1                | 2.3      | 6                       | 3                | 50.0     |
| Experimental stock..... | 49                 | 1                | 2.0      | 75                      | 53               | 70.7     |
| Total.....              | 92                 | 2                | 2.2      | 81                      | 56               | 69.1     |

\* Animals surviving the pox epidemic of 1932-33.

† Animals born after the pox epidemic of 1932-33.

epidemic. The mother had been infected with the pox about the time of their birth or shortly before, but it had not been severely ill and quickly recovered. The litter, comprising four vigorous, healthy young, had been examined carefully at frequent intervals up to the middle of March, 1933, but none of the animals in it had ever presented demonstrable signs of infection. It is seen that although the two animals under consideration have been classified as presumably immune because their birth took place during the second epidemic to which they were exposed, they were both known to have escaped infection, and actually they belong in the susceptible class. Even though one of them had been inoculated with *Tr. pallidum*, and the other was in the uninoculated breeding stock group, both contracted the disease during the third epidemic and one died.

A revision of the classification in the light of these findings indicates that every one of 90 adult animals presumably immune to the virus of

the second epidemic escaped infection during the later one. On the other hand, 58 or 69.9 per cent of 83 presumably susceptible rabbits contracted the disease. This analysis demonstrates fairly conclusively that the immunity reactions developed as a result of infection with the virus of the 1932-33 epidemic conferred an effective protection against the etiological agent of the 1933-34 epidemic, and on this basis the two diseases are immunologically related.

The morbidity rate among susceptible adult animals has been given as 69.9 per cent. Eleven of the 83 susceptible animals died with characteristic lesions at autopsy and 19 additional infected animals were sacrificed. On an extremely conservative estimate, at least one-quarter of the discarded animals would probably have died had the disease been permitted to pursue an uninterrupted course. With this correction, the mortality in the susceptible adult stock would be 15 out of a population of 83 or 18 per cent. This compares with an adult mortality of 17 per cent in the 1932-33 epidemic.

It will be recalled that animals in the epidemic room were segregated according to their use for breeding or experimental purposes. The experimental animals with their high morbidity rate were housed on racks in the south end while the breeding stock, in which the disease incidence was low, were kept in the northern section. An intervening unoccupied space separated the two groups. It would seem that the different morbidity rates in the two groups, instead of being due to a large proportion of specific immunes among the breeding stock, and the small percentage of immunes among the experimental animals, might be explained with equal merit by the hypothesis that the exciting agent had never reached the breeding animals in the northern section. That this was not the case was demonstrated by the observation that one breeding doe which was caged on a rack abutting the northern wall of the room, developed typical pox lesions and subsequently died. On this evidence it can be surmised that the virus had been disseminated throughout the room from the southern portion where its effects were first seen and came in contact with all of the animals therein.

It was at first difficult to understand the complete absence of infections in the young stock born in the epidemic room and exposed to the disease, but in the light of subsequent observations on the reaction of

the rabbit to intradermal inoculation with dermovaccine virus (7), a plausible explanation can be offered. During the course of this work it was found that young rabbits nursed by immune mothers were more refractory to inoculation with vaccinia than were young nursed by susceptible mothers. Moreover, the lowest incidence of generalized vaccinia occurred in the group comprising the nursing young of immune mothers. The evidence indicated that animals of nursing age were refractory to inoculation, and that this refractoriness was further increased when the nursing mother was itself an immune.

An application of these findings to the question under consideration points to similar conclusions. The four litters born in the epidemic room were all nursed by mothers that had recovered from the disease contracted during the second epidemic and were, therefore, immune. None of this group of 18 surviving nursing young ever presented any evidence which would lead one to suspect the presence of the disease prevailing during the third epidemic. In marked contrast to this, all of the animals in the two litters which were transferred to the epidemic room on December 18 were seriously affected and several died.

The lineage and history of these two litters are of considerable interest. Early in the second epidemic, a doe contracted the pox infection and subsequently recovered. Toward the end of this epidemic, on Jan. 12, 1933, it gave birth to a litter of five young, and so far as is known none of them ever developed the disease. One of the animals in this group of five was the mother of the first of the two litters under consideration. Since this animal itself had not contracted the disease during the second epidemic, it must be classified as a susceptible. It is highly significant that all of its young were infected during the third epidemic.

The five young animals comprising the second litter were caged with their own mother. The latter was born on May 9, 1932, and had survived the epidemic of 1932-33 as an immune. A few weeks after the doe gave birth to the litter under review, it showed signs of deterioration as evidenced by loss of weight, abdominal distension and a sluggish, listless demeanor. The poor physical condition continued from the time the young were approximately 5 weeks of age until after they were transferred to the epidemic room and developed characteristic pox lesions. Our experience with nursing does presenting the symptom complex described is that they cease nursing, and the young are forced to shift for themselves. Although in the present instance it is not known with certainty that this was the case, it is reasonable to assume that the young received little or no nourishment from their immune mother. All five came down with pox lesions during the 1933-34 epidemic.

A further point of interest connected with these two litters concerns the evidence afforded of the transmission of infection through the medium of a personal carrier. There is little doubt that infection was transmitted to the first animal of this group by some one who had handled an infected animal of the group in which the disease first appeared. Moreover, this must have occurred before infection was clinically recognizable in the animal from which the virus came as all communication was interrupted as soon as the disease was detected. It is also of interest to note that infection was first conveyed to the litter of a non-immune mother rather than to the young of the immune doe, which were handled at the same time.

TABLE II

*Nursing Litters Exposed to the Epidemic Pox Disease of 1933-34, with Number of Animals Infected in Each Litter*

| Litter No. | No. of animals | Age on Dec. 18, 1933<br>days | Immune state of mother | No. of cases of pox | Comment                                   |
|------------|----------------|------------------------------|------------------------|---------------------|---|
| X-1517     | 4              | 16                           | Immune*                | 0                   | Born in the epidemic room                 |
| X-1406     | 5              | 28                           | Immune*                | 0                   | " " " " "                                 |
| X-1351     | 2              | 34                           | Immune*                | 0                   | " " " " "                                 |
| AP-18      | 7              | 1                            | Immune*                | 0                   | " " " " "                                 |
| X-1196     | 5              | 48                           | Susceptible†           | 5                   | Transferred to the epidemic room          |
| BA-102     | 5              | 48                           | Immune*                | 5                   | " " " " "<br>Mother not nursing her young |

\* Survivor of the 1932-33 pox epidemic.

† Survivor of the 1932-33 pox epidemic, but known not to have been infected.

Table II summarizes the findings with respect to the status and reaction of the six nursing litters. None of the nursing young of four mothers immune to the causative agent of the second epidemic contracted the disease prevalent during the third epidemic. On the other hand, the infection was noted in all of the young of a susceptible doe, and also in every member of a litter of nursing age which had been weaned prematurely by their immune mother because of the mother's physical deterioration. This evidence indicates that nursing a specifically immune mother probably protects young animals from developing the lesions of a spontaneous and epidemic disease, rabbit pox.

There is some evidence to suggest that the protection afforded the



nurslings of immune mothers is transitory and is lost after weaning. The litter born on December 18 during the height of the epidemic was never demonstrably infected. Vaccination of six animals of this group with dermovaccine virus at the age of approximately 3 months, that is after weaning had occurred, gave definite positive reactions in three animals. Thus, the protective influence derived from nursing an immune mother, unlike the specific immunity acquired by recovery from actual infection, was of comparatively short duration.

The clinical features of the disease encountered during the reported epidemic were similar in most respects to those noted in the epidemic of the previous year. With the exception of the skin findings, this was true also of the lesions observed at autopsy. During the second epidemic "in early lesions the epidermis remained intact and unaltered. Pathological changes were demonstrated only in those cases associated with large necrotic areas in the corium" (3) (Fig. 2). In contrast to this was the microscopic picture of the skin lesions in the third epidemic (Fig. 1). Early lesions, involving the epidermis alone, were characterized by vacuolization and vesicle formation. The vesicles usually contained small numbers of polymorphonuclear leucocytes and strands of degenerated epithelial cells. Sometimes the lesion extended through the Malpighian layer into the papillae of the corium, both epidermis and the adjacent corium becoming involved in a necrotic process. The clinical absence of scar formation was probably due to the great frequency of skin and mucous membrane lesions limited to the epidermis.

Greene has already emphasized the numerous points of similarity between rabbit pox and human small pox. The characteristic lesions in the epidermis noted in the present epidemic were remarkably like those observed in human small pox, and this observation adds further weight to the analogy between the pox disease of man and rabbit.

The reasons for the differing locations of the microscopic lesions in the skin as observed in the two epidemics are not known. A selectivity on the part of the infecting agent, for the corium in the second epidemic and for the epidermis in the third, seems to be an untenable hypothesis in view of the similar histologic findings in the lesions of the special organs; that is, the essential lesion in both epidemics was a vascular injury involving blood vessels and lymphatics.

A more probable explanation involves a difference on the one hand, in the virulence, concentration or dosage of the two viruses, and on the other, in the composition and reactive status of the colony in the two epidemic periods.

Although no actual cross-immunity or serum neutralization tests were carried out, the available evidence from an immunological point of view indicates that the two viruses were closely related. On the basis of these observations it cannot be stated categorically that this relationship was or was not one of identity. Our previous experience has shown for instance, that an animal specifically immune to rabbit pox is refractory to inoculation with vaccine virus, both neurotropic and dermatropic strains, whereas the inoculation of a dermovaccine virus immune with rabbit pox gives an altered yet definite take. Similar findings were observed in serum neutralization experiments. The accumulated evidence thus demonstrated a qualitative relationship between the virus of rabbit pox and the virus of vaccinia, the difference being one of degree rather than of kind. The refractoriness of animals immune to the second epidemic virus when exposed to the virus of the later epidemic can be explained by a similar relationship between the two exciting agents although the disease during the reported epidemic was much more severe than ordinary vaccinia. In the absence of crucial tests, it is not known whether the two viruses were identical, but the many features common to both epidemics certainly indicate a close relationship between them. That the etiological agent of the reported epidemic might have been a variant form of dermovaccine virus derived from a passage strain, is a possibility that should be emphasized.

It is highly probable in this instance, as in the epidemic reported by Greene, (1) that an unrecognized outbreak of pox occurred in a group of animals that was not under close observation and that the infection was transported to our stocks by the caretaker in the manner indicated above. That spread of infection may occur in this manner is clearly demonstrated by our own experience. However, a number of laboratories in the Institute were working with vaccine virus at the time and despite efforts to guard against spread, the virus could very easily have been disseminated through the hallways and corridors leading to and from the animal rooms. In addition, the use of a common

nurslings of immune mothers is transitory and is lost after weaning. The litter born on December 18 during the height of the epidemic was never demonstrably infected. Vaccination of six animals of this group with dermovaccine virus at the age of approximately 3 months, that is after weaning had occurred, gave definite positive reactions in three animals. Thus, the protective influence derived from nursing an immune mother, unlike the specific immunity acquired by recovery from actual infection, was of comparatively short duration.

The clinical features of the disease encountered during the reported epidemic were similar in most respects to those noted in the epidemic of the previous year. With the exception of the skin findings, this was true also of the lesions observed at autopsy. During the second epidemic "in early lesions the epidermis remained intact and unaltered. Pathological changes were demonstrated only in those cases associated with large necrotic areas in the corium" (3) (Fig. 2). In contrast to this was the microscopic picture of the skin lesions in the third epidemic (Fig. 1). Early lesions, involving the epidermis alone, were characterized by vacuolization and vesicle formation. The vesicles usually contained small numbers of polymorphonuclear leucocytes and strands of degenerated epithelial cells. Sometimes the lesion extended through the Malpighian layer into the papillae of the corium, both epidermis and the adjacent corium becoming involved in a necrotic process. The clinical absence of scar formation was probably due to the great frequency of skin and mucous membrane lesions limited to the epidermis.

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#### EXPLANATION OF PLATE 14

FIG. 1. Section through an early lesion in the skin of the ear. The disease was contracted during the reported epidemic. The lesion is most marked in the epidermis in which vacuolization, necrosis and vesicle formation are present. There is a moderate amount of infiltration of monocytes and lymphocytes in the corium.  $\times 120$ .

FIG. 2. Section through an early lesion in the skin of the ear comparable with that in Fig. 1. The disease was contracted during the epidemic of 1932-33. The epidermis is intact, but the corium shows a marked infiltration with lymphocytes and monocytes, congestion of the blood vessels, and areas of perivascular collaring with small round cells.  $\times 120$ .

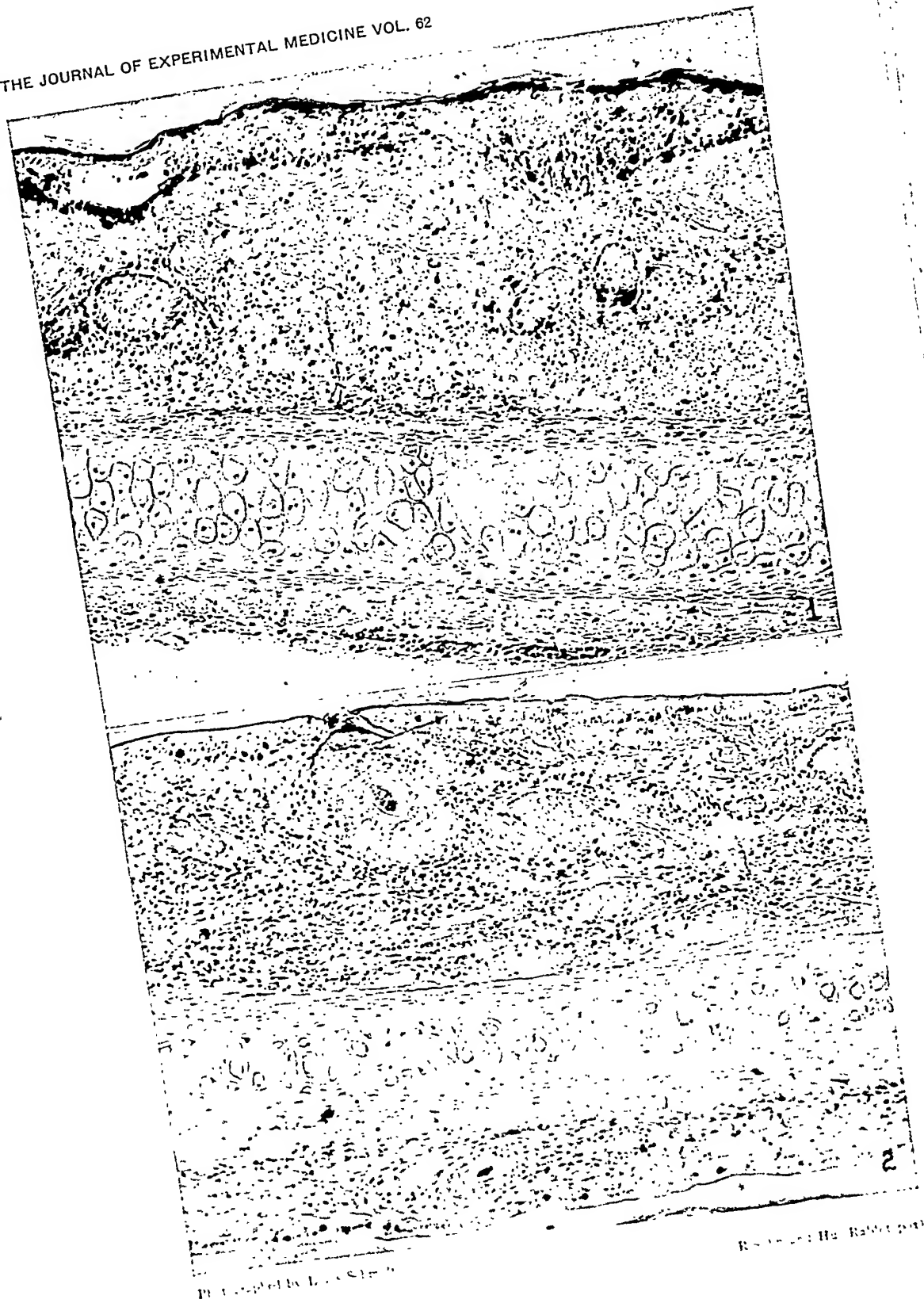
incinerator for the disposal of infected carcasses greatly increases the opportunities for spread. From the observations on the reaction of the rabbit to vaccinia, it was concluded that an animal possessing a refractory constitution sufficient to inhibit the activity of the inoculated virus may become a latent carrier. Such animals are evidently potential sources of danger and probably serve as the actual carriers of the infection. Although the presence of contagious materials is a most important factor in the production of these epidemics, it appears true that at times, and for unknown reasons, the chances of extensive spread of vaccine virus are rather remote, while at other times the virus assumes a virulence and contagiousness of an extremely high order.

#### SUMMARY

Observations on an epidemic of rabbit pox occurring in an isolated animal room during the winter of 1933-34 are reported. The clinical manifestations, consisting of a generalized papular eruption involving the skin and mucous membranes, together with blepharitis, ophthalmia, nasal discharge and lymphadenopathy were essentially similar to those noted in a pox epidemic of the previous year. This was true in general also of the pathological findings except that vacuolization, local necrosis and vesicle formation were seen in the epidermis, while in the previous year the microscopic pathology in the skin was confined to the corium. Evidence was presented indicating that the infection can be transmitted through the medium of a personal carrier, and that transmission in this manner can occur during the incubation period or before a definite diagnosis is possible. The findings also demonstrated that the etiological agents responsible for the disease reported here and that of the previous year were immunologically related, and that the immunity in recovered animals effectively persisted during the entire period for which data are available, 9 to 12 months. It appeared also that young animals suckling an immune doe were more refractory to the development of the lesions of rabbit pox than were the young of susceptible does.

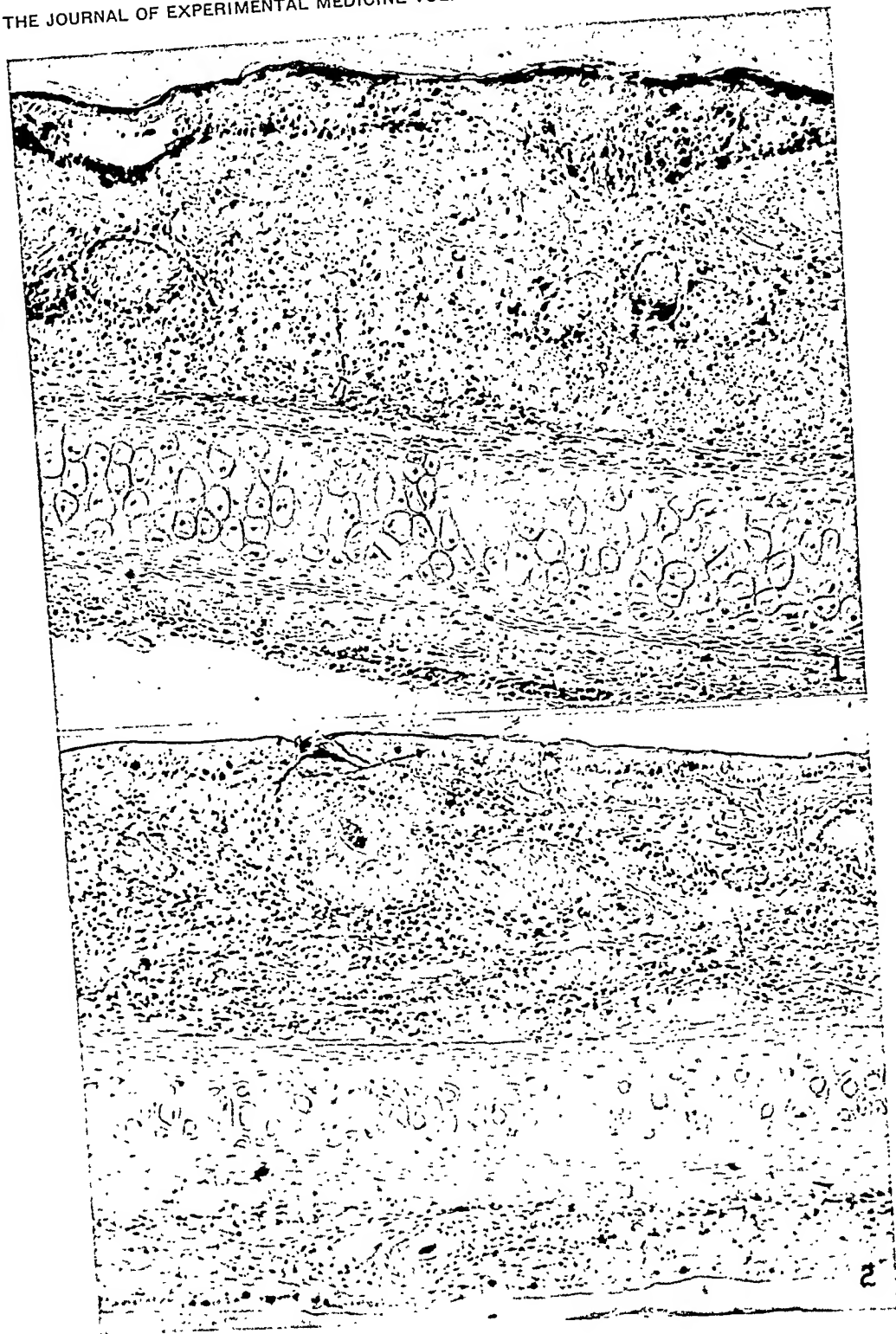
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# IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

## II. CHEMICAL ANALYSIS OF ELEMENTARY BODIES OF VACCINIA

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Through the application of recently developed methods we have been able to obtain appreciable amounts of elementary bodies of vaccinia in a relatively pure state. In view of the current findings as concerns importance of the chemical nature of viruses (1-3), it has seemed advisable to analyze some preparations of elementary bodies and to record the results.

### *Materials and Methods*

Elementary bodies of vaccinia were obtained according to the method of Craigie (4) and Parker and Rivers (5) which consists of differential sedimentation in horizontal and angle centrifuges. Suspensions of the bodies obtained in this manner (one rabbit yielded approximately 35 cc. of suspension containing about 2 mg. of dry bodies) were uniform in regard to the picture observed in stained preparations (5) and were infectious for rabbits in a dilution of  $1 \times 10^{-8}$ . Furthermore, the bacterial content of the suspensions, as determined by poured plate counts, was negligible inasmuch as there were less than 300 organisms per cc.

The suspensions of elementary bodies were frozen and dried *in vacuo*. The dried bodies were then subjected to standard qualitative tests for proteins, fats, sugars, and certain other substances. Samples of dry elementary bodies for quantitative analyses were weighed on a Kuhlmann microchemical balance sensitive to 0.001 mg. Residual moisture in the bodies was estimated by determining the loss of weight resulting from drying a sample of them in a platinum dish at 110°C. These figures were confirmed by means of drying other samples to constant weight *in vacuo* over dehydrating agents. Ash determinations were made on some of the samples by weighing the residue after ignition. The amount of protein present was estimated from the nitrogen content determined by the

Pregl (6) micro-Kjeldahl method and confirmed by a Pregl micro-Dumas combustion. The factor used to convert nitrogen to protein was 6.25. Fats were estimated by weighing the material extracted by ether from dried elementary bodies, centrifugation being used to eliminate the ether-insoluble portion.

### EXPERIMENTAL

Elementary bodies of vaccinia washed 4 times in phosphate buffer and 3 times in distilled water (5) were dried and subjected to qualitative and quantitative chemical analyses in the manner described above. The following results were obtained.

TABLE I  
*Results of Chemical Analysis of Elementary Bodies of Vaccinia*

| Analysis   | I               | II and III      | IV              | Average         |
|--|-----------------|-----------------|-----------------|-----------------|
|  | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> |
| Protein.....   | 83.38           | 82.30, 85.20    | 81.61           | 83.12           |
| Fat.....   | 10.15           | 8.95            | 6.52            | 8.54            |
| Ash.....   | 0.46            | 0.46            | 1.25            | 0.72            |
| Residual moisture.....                               | 5.37            | 5.11, 5.16      | 6.60            | 5.56            |
| Undetermined, including a trace of carbohydrate..... | 0.75            | 1.71            | 4.02            | 2.06            |

The figures for protein were calculated from the total amount of nitrogen found.

### *Qualitative Analysis*

Positive biuret, xanthoproteic, Millon, and Ehrlich's para-dimethylamidobenzaldehyde tests indicated the presence of protein. The Liebermann, Adamkiewicz, and Acree-Rosenheim tests were negative. A positive acrolein test demonstrated the presence of fats. Some preparations yielded a strongly positive Molisch reaction for the presence of carbohydrates while others exhibited a very weak one. Tests for the presence of sulfur yielded negative results. Tests for phosphorus were not made, because the elementary bodies during one stage of their preparation had been washed in a phosphate buffer solution.

### *Quantitative Analysis*

Four different batches of elementary bodies were prepared for quantitative analysis. Each lot contained approximately 20 mg.

of dry material. Two of the batches were examined separately, while the remaining two were pooled in order to obtain sufficient material for duplicate estimations of the nitrogen or protein content. The results of the analyses, summarized in Table I, indicate that elementary bodies of vaccinia contain fat, ash, carbohydrate, and nitrogen some of which is in the form of protein.

The protein in the elementary bodies was soluble in dilute alkali and to a certain extent in 70 per cent alcohol. It was only slightly soluble in water, insoluble in dilute acid, and coagulated when heated to a temperature of 65°C.

Since the concentration of sugar in the washed elementary bodies was low and relatively large amounts of material are required for quantitative determinations of carbohydrates, such determinations were not attempted. However, the results shown in Table I indicate that the carbohydrate fraction represents but a small part of the washed elementary bodies. We were surprised to find such a small amount. It occurred to us, however, that much of the sugar might have come away from the bodies during the process of purification by repeated washing and centrifugation. Consequently, we applied the Molisch test and the precipitin reaction to each of the seven wash waters obtained during the purification of a batch of the bodies. Each wash water consisted of about 50 cc. and was evaporated down to a volume of 4 cc. before the tests were made. The first wash water yielded a positive Molisch reaction in a dilution of 1:1600. The amount of sugar in the subsequent wash waters gradually decreased until a positive test was not obtained in the seventh in a dilution greater than 1:200. The results of precipitin reactions conducted with the different wash waters and antivaccinal serum roughly paralleled those of the Molisch tests in that less and less precipitinogen was found in the wash waters as purification proceeded.

#### DISCUSSION

The material analyzed by us was undoubtedly composed for the most part of elementary bodies. Furthermore, the elementary bodies either represent vaccine virus or are intimately associated with it (5). Therefore, the results of our analyses, which showed the presence of protein, fat, carbohydrate, and ash in the material examined, may

be construed as throwing light upon the chemical nature of vaccine virus or certain structures closely associated with it. It is also interesting to note that the elementary bodies were almost completely depleted of sugar by the repeated washings.

Our results do not support the idea of "protein-free" viruses, as set forth by Kligler (1), Kligler and Olitzki (2), and others, but agree closely with those of Schlesinger (3) who stated that he found in a purified coliphage, fats, an extractable carbohydrate, and nitrogen (13.2 per cent) presumably in the form of protein. Moreover, the work now described is in accord with that of Wilson Smith (7) and Ch'en (8) who have reported that they were able to obtain a specific carbohydrate from emulsions of tissues containing vaccine virus. Finally, the results of the chemical analyses of elementary bodies of vaccinia do not differ materially from those recorded for bacteria by Nencki and Schaffer (9).

#### CONCLUSION

Washed elementary bodies obtained from dermal vaccine virus contain ash, carbohydrate, fat, and nitrogen a part of which is undoubtedly in the form of protein. These components are similar to those found in bacteria and other substances of protoplasmic origin.

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## BARTONELLA BODIES IN THE BLOOD OF A NON-SPLENECTOMIZED DOG

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In the course of a series of plasmapheresis experiments on dogs, a 10.5 kg. male mongrel poodle developed an uncontrollable anemia with hemolytic and icteric plasma and urine and with many round and rod-shaped bodies in or on the erythrocytes identical to those described and named *Bartonella canis*. Inasmuch as the literature states that the spontaneous appearance of *Bartonella canis* and the successful transmission of the infection has been only in splenectomized dogs, the finding of *Bartonella* bodies in the blood of our non-splenectomized dog seems worthy of recording.

Kikuth (2, 3) and others (6, 8) demonstrated cyclical periods of anemia in splenectomized dogs associated with the presence of bodies in the erythrocytes belonging to the *Bartonella* group. These bodies showed great similarity to *Bartonella bacilliformis*, the etiological agent in Oroya fever of human beings (10) and to the *Bartonella muris* of splenectomized rats (5) and were named *Bartonella canis*. The characteristic microorganisms appeared in the blood in greatest numbers just prior to the greatest drops in the erythrocyte count and hemoglobin. The infection could be transferred to uninfected splenectomized dogs. They could not culture the microorganisms. Neosalvarsan exercised a specific influence on the infection. In this laboratory, Knutti and Hawkins (4) found *Bartonella* bodies in the blood of splenectomized gall bladder-renal fistula dogs which were exhibiting spontaneous periods of anemia associated with excessive bile pigment production. Their simple splenectomized dogs did not spontaneously show such periods of anemia but when inoculated with blood from the infected dogs showed intervals of blood destruction associated with the presence of *Bartonella* bodies. Rhoads and Miller (9), feeding a deficient black tongue-producing diet, demonstrated severe anemia in splenectomized dogs associated with the presence of *Bartonella* bodies in the blood. They were able to transmit the infection to other

splenectomized dogs fed either a normal or a deficient diet but not to non-splenectomized dogs on similar diets.

### *Methods*

Briefly, our experiments dealt with the systematic standardization of food proteins for potency in plasma protein regeneration, as previously described (1, 7). The general plan of these experiments was that blood plasma proteins were depleted by practically daily bleeding with the return of washed red cells obtained from healthy donors (plasmapheresis), so that the dogs were brought to a steady state of low plasma protein (3.6 to 4.0 per cent) and uniform plasma protein production on a given basal diet. Such dogs were excellent test subjects by which the potency of various diet factors for plasma protein regeneration could be measured by adding them to the basal diet.

On Oct. 2, 1934, Dog 34-53 was placed on a basal diet consisting of soy bean meal, Karo corn syrup, canned tomatoes, cod liver oil, cottonseed oil and a salt mixture. This diet included all the essential vitamins, furnished 75-80 calories per kilo, was readily consumed and maintained the animal at a constant weight and in good clinical condition for about 13 weeks. The initial normal plasma protein level of 7.3 per cent was lowered to 5.4 per cent by 9 weeks of this diet without plasmapheresis. Plasmapheresis was begun on Dec. 1, 1934, but after 4 weeks, it was obvious that soy bean meal was too efficient as a protein builder to make a satisfactory basal diet. The blood volume of this dog was 890 ml. and an average of 1050 ml. of blood were being removed each week without lowering the plasma protein level to the desired base line. Potatoes and bran were substituted for soy bean in the diet on Dec. 28. The red cell hematocrit had been maintained at 45-50 per cent throughout the first 4 weeks of plasmapheresis but at the close of the 5th week (Jan. 4, 1935) it had dropped to 41.3 per cent in spite of replacement of red cells ordinarily adequate to maintain the normal level. The next day the plasma showed rather marked hemolysis and the animal vomited bloody mucus. Following each subsequent exchange of blood the plasma and urine grew progressively more jaundiced, the mucous membranes became pale and yellow, the diet was only partially consumed and vomiting was frequent. Occult blood was found in the urine. The hematocrit was 33.4 per cent on Jan. 3. Plasmapheresis was discontinued, due to the unfavorable reactions; and kidney, liver and Lextron (primary and secondary anemia liver fractions plus iron) were incorporated in the diet to aid in hemoglobin regeneration. During the 40 days of plasmapheresis, 5354 ml. of whole blood were removed from the dog. This contained 3214 ml. of plasma bearing 143.3 gm. of protein. The plasma protein level was lowered to an average of 4.14 per cent for the last week.

On Jan. 14 the plasma protein level had climbed to 5.1 per cent but the hemato-

crit had dropped to 19.4 per cent. The leucocyte count was 27,600 with 80 per cent polymorphonuclears. Twenty normoblasts, several megaloblasts and many large polychromatophilic erythrocytes, possibly reticulocytes, were seen in counting 100 leucocytes. A striking finding in the blood smears was many small coccoid bodies and beaded rods of varying lengths staining blue by Wright's method in or possibly on the erythrocytes, sometimes singly but often several to a cell. These bodies were morphologically indistinguishable from those described by various investigators as *Bartonella canis*. They were found on 3 consecutive days. No blood exchanges had been made for 6 days due to the reaction following each but with the hematocrit at 18.5 per cent on Jan. 15, a whole blood transfusion was considered imperative and 198 ml. were given. The hematocrit was 23.4 per cent on the following day and the *Bartonella* bodies were still present. On Jan. 16, 165 mg. of neoarsphenamine or the equivalent of 15 mg. per kilo weight were injected intravenously. The smears were negative the following day and have remained so over a period of 15 weeks. 3 days after the apparent sterilization the hematocrit had climbed to 32.5 per cent and the plasma was only slightly icteric. 25 ml. of washed red cells from a donor dog were injected to observe the reaction. The animal vomited, occult blood appeared in the urine and the hematocrit dropped to 30.6 per cent showing that there still remained an unfavorable reaction towards the injection of erythrocytes even though the *Bartonella* infection had apparently disappeared. Subsequently the hematocrit has climbed to 42 per cent, the dog appears to be in perfect health and is in the animal colony awaiting further work on the hemolytic problem. An exploratory laparotomy performed on Apr. 26 showed normal appearing abdominal viscera including a spleen measuring 14 x 5 x 1-2 cm.

Just prior to the neoarsphenamine sterilization on Jan. 16, 10 ml. of blood from Dog 34-53 was injected intravenously into splenectomized Dog 33-353 whose blood smears were negative for *Bartonella* bodies and whose red cell hematocrit was 40.4 per cent with water clear plasma. 3 days after the injection the hematocrit was 35 per cent, the plasma was distinctly icteric and occasional coccoid bodies and short, slender rods were seen in the erythrocytes stained by Wright's method. By the 9th day the infection was heavy, with *Bartonella* bodies present in practically every red cell and the hematocrit was 32 per cent. The following day (Jan. 25) the hematocrit was 27 per cent. The bodies could only be found after careful searching and remained scarce for 3 days. On Jan. 28, the microorganisms appeared in great numbers in the erythrocytes and the hematocrit stood at 30 per cent. 3 days later the hematocrit dropped to 24 per cent with increased icterus of the plasma and the bodies were again difficult to find. Having satisfactorily demonstrated *Bartonella* infection in splenectomized Dog 33-353, a single dose of neoarsphenamine (15 mg. per kilo) was injected intravenously with apparent sterilization. The hematocrit climbed back to 38.5 per cent in 3 weeks, the plasma lost its icteric color and no *Bartonella* bodies were demonstrated in multiple examinations. Howell-Jolly bodies were frequently encountered in the erythrocytes



during the anemic periods. The dog was in good physical condition throughout this period of transmitted infection and is still in our animal colony.

#### DISCUSSION

Inasmuch as *Bartonella* infection has been reported only in splenectomized dogs and with but few exceptions in splenectomized rats, the spleen has naturally been assumed to exert a protective action. Kikuth thinks that a latent infection may be held in check by the reticulo-endothelial system so that if this system is interfered with by the loss of a great portion of it, such as the spleen, the power is lost. Wills and Mehta (11) produced severe anemia in intact rats by feeding diets deficient in vitamins A and C and demonstrated *Bartonella muris* in their red cells in great numbers. They raised the question as to the possibility of the deficient diet having a specific action on the reticulo-endothelial system rendering the animal peculiarly liable to infections. We are at a loss to suggest a plausible explanation for the infection in our dog since the spleen was intact and all essential vitamins were adequately furnished in the diet. It is possible that the spleen elaborates some substance which is liberated into the circulating blood which has an inhibitory effect upon the *Bartonella* infection. Knutti and Hawkins (4) have shown that spleen extract feeding in *Bartonella* infected, splenectomized, bile fistula dogs appeared to have an inhibiting effect upon the periods of anemia and bile pigment overproduction. Our Dog 34-53 may have carried a latent infection of *Bartonella* which was activated by the steady loss of large amounts of blood with the depletion of plasma protein. Since this animal was losing more blood each week than its total blood volume, it may well have been losing some protective substance elaborated in the spleen. It may also have been inoculated with the infection from one of the donor dogs furnishing red cells for the exchanges. However, these same donor dogs have been in use in our plasmapheresis colony for several years without the previous appearance of *Bartonella* bodies.

The cyclical periods of anemia with the appearance of the *Bartonella* bodies in greatest numbers just prior to the greatest drops in the hematocrit are well illustrated in the inoculated splenectomized Dog 33-353. The association of the rapidly developing anemia and *Bartonella* bodies in non-splenectomized Dog 34-53 is also suggestive

but not conclusive evidence of their etiological relationship. This becomes more doubtful as one considers the hemolysis and fall in the hematocrit following the injection of only 25 ml. of washed red cells after the apparent sterilization with neoarsphenamine. Since the hemolytic episode with this dog, two other dogs, after several weeks of plasmapheresis, suddenly developed severe bouts of hemolysis, jaundice and anemia with hematocrits as low as 7 per cent, but *Bartonella* bodies were not demonstrated. Neoarsphenamine did not alter the course in one of these dogs and was not tried on the other. Both animals have recovered but are no longer suitable for plasmapheresis, since this hemolytic phenomenon has been repeatedly observed in them following the injection of washed erythrocytes. Other dogs tolerate plasmapheresis over long periods. One animal was exchanged for 41 weeks with 55,580 ml. of blood removed in 202 exchanges (7).

#### SUMMARY

A non-splenectomized dog, on a vitamin-adequate basal diet, in the course of a plasmapheresis experiment, developed an uncontrollable anemia associated with the presence of bodies in or on the erythrocytes, indistinguishable from the descriptions of *Bartonella canis*. The normal plasma protein level of 7.3 per cent was reduced to 4.1 per cent by diet and the removal of 5354 ml. of whole blood in 33 bleedings. The *Bartonella* infection was transferred to a splenectomized dog by an intravenous injection of whole blood. Each animal was apparently sterilized by one injection of neoarsphenamine equivalent to 15 mg. per kilo weight. It is possible that the spleen liberates some substance into the blood stream which has an inhibitory effect upon a latent *Bartonella* infection and that this protective substance was diminished by the many bleedings associated with the lowering of plasma proteins in the non-splenectomized dog and was lacking in the inoculated splenectomized dog.

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# THE PROTECTIVE ACTION OF TYPE I ANTIPNEUMOCOCCUS SERUM IN MICE

## I. THE QUANTITATIVE ASPECTS OF THE MOUSE PROTECTION TEST

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The demonstration of the protective or curative action of an antibacterial serum such as antipneumococcus serum is conditioned not alone by the antibody content but in a large measure by as yet ill defined characteristics of the animal. It is well recognized that the action of the specific antibody alters certain qualities of the bacterium or its products. The eventual outcome, however, is in large measure dependent upon the various non-specific factors of the animal body. The evaluation of the latter is a particularly complex and difficult problem. For example, it is known that unidentified characteristics of various animal species are of great importance. Then, too, the rôle of genetic factors within a species is clearly appreciated. Moreover, within the same genetic strains, certain physiological variables appear to be of the greatest significance. When the latter can be correlated with the constitutional response they are termed "individual host factors."

Previous communications (1, 2) have dealt with the nature and influence of the individual host factors of the rabbit with reference to the curative and protective actions of specific antipneumococcus serum. Under relatively controlled genetic conditions the end-result appeared in any given instance to be conditioned by the quantitative interrelationships of four variables: (a) the number of infecting organisms, (b) the amount of antiserum (in effect the amount of antibody), (c) the weight of the animal, and (d) the number of circulating white blood cells. No evidence was obtained to show that either the weight or the number of cells is other than a reflection of a more complex physiological system. They serve, however, as indices, and, as such, they express the quantitative aspects of non-specific host resistance in the rabbit.

The present series of papers deals with the problem of the host factors which are concerned in the protective action of antipneumococcus serum in white mice. As in any study of this kind, it was first necessary to determine the general quantitative characteristics of the mouse protection test in order to learn what combinations of serum and culture might be used advantageously in the evaluation of the rôles played by the various host factors.

The mouse protection test for the evaluation of the potency of specific antipneumococcus serum has long been used and has for many years been the subject of controversy, particularly since it is a method for standardization. The earlier methods, such as those of Neufeld (3), and of Cole and his associates (4), were used to establish minimal requirements for the protective capacity of a serum. The latter method, as later adopted by the United States Hygienic Laboratory (5), was based on the simultaneous injection of culture and serum, and required that 0.2 cc. of serum should protect two of three mice when injected together with 0.1 cc. of a culture of a virulence such that 0.000,001 cc. would bring about the death of normal controls. It was recognized that a considerable individual variation among mice did not permit the more stringent requirement of 100 per cent protection.

Subsequent improvements in culture media and the knowledge of bacterial dissociation have made it possible to obtain cultures of *Pneumococcus* containing at least 100 times as many virulent organisms as did cultures at the time these protective tests were first described. Since the earlier requirements were based on the amount of culture rather than on the number of minimal fatal infective doses, the result has been to increase the severity of the test.

A second development within the past few years has been the introduction of various forms of concentrated sera. As will be shown in the text, these concentrated preparations, although high in antibody content, give misleading results with the original minimal potency tests.

In view of these facts, Felton (6) has developed a second type of protection test designed to give a quantitative estimation of serum potency. This test consists essentially in the simultaneous injection of varying amounts of antiserum together with a fixed quantity of culture, usually 100,000 minimal fatal infective doses. The end-point or point of quantitative evaluation is taken as the smallest quantity of serum which under these conditions will protect two-thirds of the mice. This last requirement again recognizes the variation among individual mice. Although this system of titration is now widely used, the results are not as quantitative as might be desired, since it is often difficult for two or more workers to arrive at the same estimation of potency on a given sample of serum except by the use of very large numbers of mice.

## EXPERIMENTAL

*Mice.*—Female white mice of the Rockefeller Institute strain were used throughout. These mice were maintained on a diet of bread and milk. This strain, while highly inbred over a long period of years, is fairly representative of the type of mice in general use for protection tests.

*Culture.*—The original Neufeld strain of Type I Pneumococcus was used in all of these experiments. Cultures were maintained in rabbit blood broth and possessed a virulence such that 0.000,000,01 cc., given intraperitoneally, invariably produced fatal infection.

*Sera.*—Type I antipneumococcus horse sera were used in these experiments.<sup>1</sup>

*Injection.*—The exact details of each experiment are described in the respective protocols. The general method was to dilute the serum and culture so that the desired quantity of each would be contained in 0.5 cc.; the serum was diluted in saline, the culture in broth. Serum and culture were mixed in the same syringe before injection. The minimal period of observation of the test mice was 6 days.

*The Protective Action of Antipneumococcus Serum When Injected  
Simultaneously with Culture*

The first experiment deals with the comparative protective actions of Type I antipneumococcus horse and rabbit sera when injected intraperitoneally with various amounts of Type I pneumococcus broth culture. These tests are essentially elaborations of the test originally employed in this laboratory. The results are shown in Text-fig. 1.

Dilutions of an 18 hour blood broth culture of Type I Pneumococcus were made in broth so that designated amounts ranging from 0.05 cc. to 0.4 cc. were contained in 0.5 cc. Dilutions of the antipneumococcus sera were made in saline so that amounts ranging from 0.001,562,5 cc. to 0.4 cc. were contained in 0.5 cc. Both horse and rabbit antipneumococcus sera had comparable agglutinin titers. The mice weighed from 18.5 to 21 gm.

This particular lot of antipneumococcus *rabbit* serum, within the range of amounts used, protected all mice against 0.05 cc. of culture. Against 0.1 cc. of culture complete protection was obtained with 0.05 cc. of serum and with larger amounts. With smaller amounts of serum the results were irregular, until with 0.003,125 cc. all mice died. Against 0.2 cc. of culture, protection was irregular throughout, and when 0.4 cc. of culture was used no protection was demonstrated.

With this lot of antipneumococcus *horse* serum all mice were protected against 0.05 cc. of culture by 0.1 cc., 0.05 cc., and 0.025 cc. quantities. With amounts of serum greater or smaller than these, at first irregular, and finally completely

<sup>1</sup> These sera were furnished through the courtesy of Dr. Augustus Wadsworth of the Division of Laboratories of the New York State Department of Health.

negative, results were observed. Similar results were obtained with varying amounts of serum against 0.1 cc. of culture, although here complete protection was obtained only with 0.05 cc. and 0.025 cc. amounts of serum. With 0.2 cc. of culture the results were irregular throughout except that with the larger amounts of serum no protection was afforded. No protection was obtained with any amount of serum when 0.4 cc. of culture was used.

| Amount of serum (cc.) | Source of immune serum  |     |     |      |       |     |     |      |
|-----------------------|-------------------------|-----|-----|------|-------|-----|-----|------|
|                       | Rabbit                  |     |     |      | Horse |     |     |      |
|                       | Amount of culture (cc.) |     |     |      |       |     |     |      |
|                       | 0.4                     | 0.2 | 0.1 | 0.05 | 0.4   | 0.2 | 0.1 | 0.05 |
| 0.4                   | ●●●                     | ●○○ | ○○○ | ○○○  |       | ●●● | ●●● | ●●●  |
| 0.2                   | ●●●                     | ○○○ | ○○○ | ○○○  | ●●●   | ●●● | ●○○ | ●○○  |
| 0.1                   | ●●●                     | ●●○ | ○○○ | ○○○  | ●●●   | ●○○ | ●○○ | ○○○  |
| 0.05                  | ●●●                     | ●○○ | ○○○ | ○○○  | ●●●   | ●○○ | ○○○ | ○○○  |
| 0.025                 | ●●●                     | ●●● | ●○○ | ○○○  | ●●●   | ●○○ | ○○○ | ○○○  |
| 0.0125                | ●●●                     | ●○○ | ○○○ | ○○○  | ●●●   | ●●● | ●○○ | ●○○  |
| 0.00625               |                         | ●●● | ●○○ | ○○○  |       | ●○○ | ●○○ | ●○○  |
| 0.003125              |                         |     | ●●● | ○○○  |       | ●○○ | ●●● | ●●●  |
| 0.00156               |                         |     |     |      |       | ●●● | ●●● | ●●●  |

●—Death

○—Survival

TEXT-FIG. 1. Protection tests with Type I antipneumococcus horse and rabbit sera. Serum and culture administered simultaneously. Type I pneumococcus culture possessed a virulence such that 0.000,000,01 cc. produced fatal infection in controls.

These results, entirely typical in our experience, clearly point to certain well defined characteristics of the protection test. These may be listed as follows:

1. The results show a considerable amount of irregularity. Thus, in many instances in which three mice received exactly the same amounts of serum and culture, some died whereas others survived. This irregularity is ascribed to variations in the capacities of the individual animals to utilize the protective qualities of the serum.

2. With very small amounts of serum no protection is obtained against relatively large amounts of culture. Here the amount of antibody is obviously inadequate in proportion to the number of infective organisms. The minimal amount of serum below which no

protection can be demonstrated with a given amount of culture is designated as the "limiting titer zone." A complete description of this phase will be presented later in this paper.

3. There appears to be a limiting maximum amount of culture against which protection can be demonstrated. Thus, with both horse and rabbit immune sera, some protection was obtained against 0.2 cc. of culture, but all mice died when 0.4 cc. of culture was injected. Neufeld has designated the upper limit of culture against which protection can be obtained with a given amount of immune serum as the *Schwellenwert* (7). In the present paper the term will be used in a more general sense as referring to the maximum amount of culture against which protection can be demonstrated irrespective of the amount of serum.

4. With the antipneumococcus rabbit serum, the protection with 0.4 cc. of serum was as good as that with 0.05 cc. On the other hand, with 0.4 cc. of antipneumococcus horse serum, no protection was obtained. This characteristic failure of large amounts of antipneumococcus horse serum to afford protection is termed the "prozone." Evidence of its existence is to be found in the protocols of many workers (*cf.* Yosiooka (8), Felton (6), Sobotka and Friedländer (9)).

5. These limiting quantitative characteristics serve to define a zone of optimal protection.

#### *Comparative Protection Tests with Various Lots of Antipneumococcus Horse Sera*

Protection tests have been carried out with several lots of Type I antipneumococcus horse serum. For purposes of comparison, protection tests on three of these lots are shown in Table I. The results of agglutinin titrations with the same sera are shown in Table II.

It will be noted that serum Lot A gave definite agglutination in a dilution as high as 1-64; Lot B, 1-256; and Lot C, a concentrated preparation, in a final dilution of 1-512. Assuming that the antibody content of these sera actually range in this order, it is of interest to examine the results of the protection tests presented in Table I. Each of the three sera show the characteristic prozone of non-protection, the protective zone, and a limiting titer zone. For each serum there is a particular optimal amount which appears to give the highest



degree of protection. Thus, Lot A gave maximum protection with 0.2 cc., Lot B with 0.05 cc., and Lot C with 0.025–0.0125 cc. It will be observed that these optimal amounts are inversely proportional

TABLE I

*Comparison of Protection Tests with Three Lots of Antipneumococcus Horse Sera (Type I)*

Serum and culture injected simultaneously. Each animal received the equivalent of 0.1 cc. of an 18 hour blood broth culture of Type I Pneumococcus.

| Amount of serum<br><br>cc. | Type I antipneumococcus horse serum |       |       |
|----------------------------|-------------------------------------|-------|-------|
|                            | Lot A                               | Lot B | Lot C |
| 0.4                        | D D S S                             | D D D | D D D |
| 0.2                        | D S S S                             | D D S | D D D |
| 0.1                        | D D S S                             | D S S | D D D |
| 0.05                       | D D S S                             | S S S | D D S |
| 0.025                      | D D D S                             | D S S | S S S |
| 0.0125                     | D D D S                             | D D S | S S S |
| 0.00625                    | D D D D                             | D D S | D S S |
| 0.003125                   |                                     | D D D | D D D |
| 0.0015625                  |                                     | D D D | D D S |

D = death.

S = survival.

TABLE II

*Agglutinin Titrations of Three Lots of Type I Antipneumococcus Serum*

Titrations against a washed heat-killed suspension of Type I pneumococci.

| Sera       | Final dilution of serum |      |      |       |       |       |        |
|------------|-------------------------|------|------|-------|-------|-------|--------|
|            | 1-16                    | 1-32 | 1-64 | 1-128 | 1-256 | 1-512 | 1-1024 |
| Lot A..... | ++++                    | ++++ | ++±  | ±     | —     | —     | —      |
| Lot B..... | ++++                    | ++++ | ++++ | ++++  | ++    | —     | —      |
| Lot C..... | ++++                    | ++++ | +++  | +++   | ++±   | ±±    | ±      |

to the agglutination titers, in that the higher the titer the smaller is the amount of serum which gives optimal protection. Likewise, the prozone becomes more marked the higher the agglutination titer of the serum. Repetitions of these tests have shown that the "optimal

protective amount" is a definite characteristic of each lot of antipneumococcus horse serum.

Each of these lots of immune serum has been used clinically in the treatment of Type I lobar pneumonia. Lots B and C gave excellent results, but Lot A proved less efficient therapeutically. It may be pointed out that of these three lots of antipneumococcus horse sera the only one which would pass the original minimal potency requirement is Lot A.

*Results Obtained by the Injection of Immune Serum 18 Hours before Infection*

Neufeld (3) has regularly followed the practice of injecting the immune serum several hours previous to the time of infection. It is of course well known that in the case of certain infective organisms, as, for example, the hemolytic streptococci of Group A (Lancefield), this method must be used in order to demonstrate the protective action of immune sera. A series of experiments was therefore undertaken to learn if the earlier administration of immune serum might exalt its protective qualities.

Mice were injected with varying amounts of antipneumococcus horse serum and 18 hours thereafter were infected with varying numbers of pneumococci. The results of this experiment are shown in Table III together with comparable data on a series in which serum and culture were injected simultaneously.

From these results it will be noted that with the "delayed" method of infection the prozone is entirely eliminated. In other experiments, not shown in the table, as much as 0.8 cc. of serum was administered several hours prior to infection and yet no prozone was demonstrable. Although this procedure abolished the prozone, the limiting titer zone was altered so that the amount of serum required to give protection was larger.

The optimal protective amount of serum also differs with different methods of conducting the protection test. With 0.1 cc. of culture all mice were protected with 0.2 cc. of serum by the delayed method, whereas four of six died by the simultaneous method. On the other hand, with 0.025 cc. of serum and 0.1 cc. of culture, three of four mice died with delayed infection whereas all survived by the simultaneous

method. Thus, the optimal protective amount is smaller with the simultaneous method than with the delayed method, and in a certain sense it may be said that the efficiency of the serum appears to be greater with the former method.

A shift in *Schwelienwert* was also produced, for it may be noted that some mice were protected against infection with 0.4 cc. of culture by the delayed method, whereas all died by the simultaneous method.

On the basis of the data presented, it is not possible to correlate the results obtained by the two methods. However, certain inferences may be drawn. It seems altogether likely that 18 hours after serum

TABLE III

*Results Obtained by Simultaneous Injection of Serum and Culture as Compared with Those Obtained by the Administration of Serum 18 Hours Previous to Infection*

| Culture          | Serum injected 18 hrs. in advance of culture |         |         |          | Serum and culture injected simultaneously |         |         |          |
|------------------|--|---------|---------|----------|---|---------|---------|----------|
|                  | 0.4 cc.                                      | 0.2 cc. | 0.1 cc. | 0.05 cc. | 0.4 cc.                                   | 0.2 cc. | 0.1 cc. | 0.05 cc. |
| <i>Serum cc.</i> |  |         |         |          |   |         |         |          |
| 0.2              | DD SS  | D SSS   | SSSS    | SSSS     | DDD                                       | DDDDD S | DDDD SS | DDD SSS  |
| 0.1              | DDD S  | D SSS   | D SSS   | SSSS     | DDD                                       | DDDD SS | DDD SSS | SSSSSS   |
| 0.05             | DDDD   | DDD S   | D SSS   | SSSS     | DDD                                       | DD SSSS | SSSSSS  | SSSSSS   |
| 0.025            | DDDD   | DDD S   | DDD S   | SSSS     | DDD                                       | DDD SSS | SSSSSS  | SSSSSS   |

D = death.

S = survival.

injection a large proportion of the antibody is no longer present in effective form in the peritoneum, and that this may account for the shift in the optimal amount of serum. Reasoning on this basis, it would appear that about seven-eighths of the effective antibody has been lost during the interval. Entirely similar findings resulted from comparable titrations by the Felton technic (6). On the basis of this conception, it would seem that protective action of serum administered with the simultaneous method is much more efficient in that smaller amounts of antibody suffice. On the other hand, this interpretation does not account for the increased protection against larger amounts of culture when serum is administered in advance, that is to say, for

the increased value of the *Schwellenwert*. If, however, one reasons from other experiences, this result might be expected in view of the fact that foreign protein in the peritoneum of the mouse acts as a stimulus to the cellular system.

*Protection Test in Mice Which Had Received a Previous Injection of a Non-Specific Agent*

In order to test the foregoing hypothesis, a series of protection tests was carried out in which serum and culture were injected simultane-

TABLE IV

*The Protective Action of Type I Antipneumococcus Horse Serum in Normal as Compared with Nucleinate Prepared Mice*

Serum and culture injected simultaneously. Each animal received the equivalent of 0.1 cc. of an 18 hour blood broth culture of Type I Pneumococcus. Each animal in the column marked Prepared had received an intraperitoneal injection of 0.5 cc. of 5 per cent sodium nucleinate 18 hours prior to the injection of serum and culture.

| Amount of serum | Normal mice | Prepared mice |
|-----------------|-------------|---------------|
| cc.             |             |               |
| 0.4             | D D D       | S S S S S     |
| 0.2             | D D S       | S S S S S     |
| 0.1             | D S S       | S S S S S     |
| 0.05            | S S S       | S S S S S     |
| 0.025           | D S S       | S S S S S     |
| 0.0125          | D D S       | S S S S S     |
| 0.00625         | D D S       | D D D D D     |

D = death.

S = survival.

ously into animals which had received a previous injection of a non-specific irritant. The results of one experiment of this order are shown in Table IV.

The irritant used in this experiment was sodium nucleinate prepared from yeast nucleic acid. It had been determined that this agent brings about a rapid increase in the number of cells in the peritoneum beginning at 4 to 6 hours after injection. The cells were found to persist in increased numbers for 2 to 3 days. In the experiment cited each animal received an intraperitoneal injection of 0.5 cc. of a 5 per cent solution of sodium nucleinate. 18 hours thereafter the animals of



It will be noted that with any given amount of culture the highest survival rate occurred with 0.025–0.05 cc. of serum. With smaller or greater amounts of serum progressively fewer mice survived. Furthermore, it will be noted that with any particular amount of serum the survival rates progressively increase as the amount of culture is decreased. Thus, the results form a definite fan-like pattern with a pivotal point of complete protection and radiating therefrom many axial lines of progressively diminishing protection.

These results serve to emphasize the four general characteristics of the mouse protection test which have been previously pointed out;

TABLE V

*Survival Rates with Various Combinations of Antipneumococcus Horse Serum and Culture*

| Amounts of<br>immune serum<br>(Lot D)<br><br>cc. | Amounts of culture |         |         |          |                     |
|--|--------------------|---------|---------|----------|---------------------|
|  | 0.4 cc.            | 0.2 cc. | 0.1 cc. | 0.05 cc. |                     |
| 0.4  |                    | 0       | 5       | 0        | } Per cent survival |
| 0.2  | 0                  | 14      | 26      | 60       |                     |
| 0.1  | 0                  | 13      | 41      | 80       |                     |
| 0.05   | 9                  | 33      | 73      | 100      |                     |
| 0.025  | 0                  | 45      | 64      | 100      |                     |
| 0.0125   | 0                  | 17      | 48      | 83       |                     |
| 0.00625  | 0                  | 0       | 30      | 33       |                     |
| 0.003125   |                    | 0       | 0       | 17       |                     |
| 0.0015625  |                    | 0       | 0       | 0        |                     |

*viz.*, the prozone, the *Schwellenwert*, the limiting titer zone, and the optimal protective amount of serum. The irregularity in each group of mice can only be ascribed to variations in individual host factors.

The second part of this experimental analysis deals with the results of mouse protection tests involving progressively diminishing amounts of both culture and serum, and particularly concerns the limiting titer zone, previously defined as the smallest amount of serum which will protect mice against a given amount of culture. This end-point is obviously not solely dependent upon amounts of either serum or culture, but rather upon the quantitative relationships of one to the other. It might be expected that if protection is predicated by a

this series together with normal control mice received injections of varying amounts of antipneumococcus horse serum together with 0.1 cc. of culture.

The results with the series of normal mice show the characteristic prozone in which larger amounts of serum failed to afford protection. With "prepared" mice, however, the prozone was completely eliminated in that all animals receiving 0.0125 cc. or more of serum survived. In other protection tests of this type the limiting titer zone has not been so sharp as in the experiment recorded, although as a rule the prozone has been greatly reduced or completely eliminated. With certain lots of high-titered concentrated sera it has not been possible to abolish entirely the prozone phenomenon by preliminary injection of sodium nucleinate.

In other experiments it was found that the previous preparation of mice also altered the value of the *Schwellenwert*. For example, prepared mice were regularly protected against 0.4 cc. of culture although in normal mice this result was rarely achieved.

These results indicate that both the prozone and the *Schwellenwert* can be modified or shifted as a result of the previous administration of a non-specific agent. It is probable that changes in the number and character of the cells in the peritoneum are responsible for these results.

### *The Quantitative Aspects of the Mouse Protection Test*

An extensive study has been made of certain quantitative aspects of the mouse protection test with antipneumococcus horse serum. For the present purposes the results with one lot of serum have been divided into two sections. The first deals with a series of graded combinations of serum and culture involving large amounts of the latter, the second with the extension of the limiting titer zone in a range involving progressively diminishing amounts of both serum and culture. The results in the first section are shown in Table V.

Amounts of Type I Pneumococcus broth culture varying from 0.05 cc. to 0.4 cc. were injected intraperitoneally together with amounts of immune serum ranging from 0.001,562,5 cc. to 0.4 cc. The results are shown in terms of percentage survival. The number of mice averaged seventeen for each combination of serum and culture.

It will be noted that with any given amount of culture the highest survival rate occurred with 0.025–0.05 cc. of serum. With smaller or greater amounts of serum progressively fewer mice survived. Furthermore, it will be noted that with any particular amount of serum the survival rates progressively increase as the amount of culture is decreased. Thus, the results form a definite fan-like pattern with a pivotal point of complete protection and radiating therefrom many axial lines of progressively diminishing protection.

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|---------------------------------------|--------------------|---------|---------|----------|-------------------|
|                                       | 0.4 cc.            | 0.2 cc. | 0.1 cc. | 0.05 cc. |                   |
| cc.                                   |                    | 0       | 5       | 0        |                   |
| 0.4                                   |                    | 14      | 26      | 60       |                   |
| 0.2                                   | 0                  | 13      | 41      | 80       |                   |
| 0.1                                   | 0                  | 33      | 73      | 100      |                   |
| 0.05                                  | 9                  | 45      | 64      | 100      |                   |
| 0.025                                 | 0                  | 17      | 48      | 83       |                   |
| 0.0125                                | 0                  | 0       | 30      | 33       |                   |
| 0.00625                               | 0                  | 0       | 0       | 17       |                   |
| 0.003125                              |                    | 0       | 0       | 0        |                   |
| 0.0015625                             |                    |         |         |          |                   |
|                                       |                    |         |         |          | Per cent survival |

viz., the prozone, the *Schwellenwert*, the limiting titer zone, and the optimal protective amount of serum. The irregularity in each group of mice can only be ascribed to variations in individual host factors.

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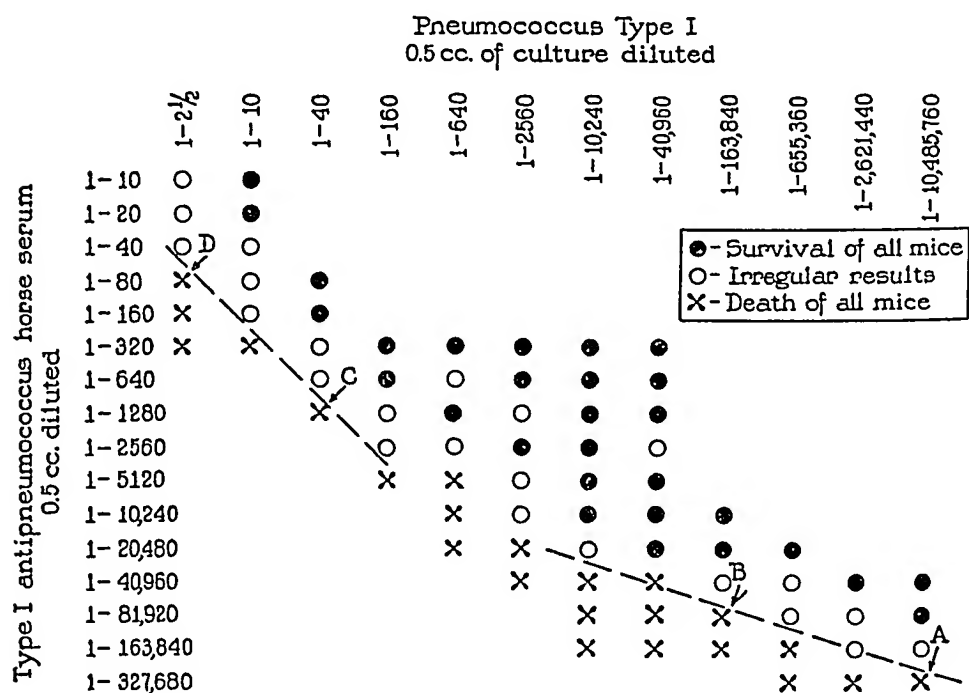
| Amounts of<br>immune serum<br>(Lot D)<br><br>cc. | Amounts of culture |         |         |          |                   |
|--|--------------------|---------|---------|----------|-------------------|
|  | 0.4 cc.            | 0.2 cc. | 0.1 cc. | 0.05 cc. |                   |
| 0.4  |                    | 0       | 5       | 0        | Per cent survival |
| 0.2  | 0                  | 14      | 26      | 60       |                   |
| 0.1  | 0                  | 13      | 41      | 80       |                   |
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| 0.00625  | 0                  | 0       | 30      | 33       |                   |
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| 0.0015625  |                    | 0       | 0       | 0        |                   |

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proportional equation between antibody molecules and infective organisms, it should be possible to demonstrate a linear interdependence between them.

In order to study this question, a large series of mice was injected with varying amounts of serum together with varying amounts of culture; the serum was progressively diluted by multiples of two, the culture by multiples of four. The results are shown graphically in Text-fig. 2.



TEXT-FIG. 2. Schematic representation of results of mouse protection test involving progressively diminishing amounts of both serum and culture.

For each of these combinations of serum and culture, a minimum of three mice was used. It is realized that the number of mice used to test each combination is not great enough to permit of rigid quantitative analysis. This deficiency, however, is to some extent compensated by the supporting results in adjacent series.

When graphically plotted as in Text-fig. 2, the results tend to arrange themselves in three distinct zones: a zone of complete protection, a

second zone of irregular protection, and a third of complete lack of protection.

Two broken lines have been inserted in the chart to show the general position of the boundary between the zones of protection and non-protection. Attention may be drawn to the slopes of these lines. Line AB indicates that in this range a fourfold increase in the amount of serum permits of a 64-fold increase in the amount of culture. On the other hand, line CD indicates that with a fourfold increase in the amount of serum the amount of culture can only be increased by the same multiple.

Any attempt to explain these results must take into account the fact that the organisms against which it is desired to protect the animal are living and multiplying biological units. It is known that when large numbers of pneumococci are injected, multiplication begins within 30 minutes. On the other hand, complete phagocytosis and bacterial destruction are not accomplished for 4 hours or longer. Therefore, when large numbers of pneumococci are injected their number has been many times augmented before bacterial destruction has been completed. Hence, the animal to be protected must overcome and destroy not merely the number of bacteria originally introduced, but their numerous progeny as well. From the evidence now available, it would seem likely that when extremely few pneumococci are injected the lag phase preceding bacterial multiplication is prolonged, and if this be of sufficient length, bacterial destruction may supervene before any cell division occurs. When amounts of serum and culture are properly chosen in order to illustrate this point the animal is confronted only with the number of pneumococci originally injected. It is in exactly this range that the maximum efficiency of the immune serum is demonstrable.

#### DISCUSSION

The present paper deals with the quantitative aspects of the mouse protection test as applied to antipneumococcus horse and rabbit sera. The test has been subjected to quantitative analysis and both the dosage of protective serum and the number of infecting organisms have been varied through wide limits.

The quantitative characteristics of the mouse protection test for

the evaluation of antipneumococcus sera may be summarized as follows:

1. *The Limiting Titer Zone.*—The smallest amount of immune serum which will protect a mouse against a given quantity of culture is apparently related both to the number of virulent organisms and to the antibody content of the serum. The evidence presented tends to show that this relationship is not strictly one of multiple proportions. In this connection it is a matter of considerable interest that specific protection can be obtained with an amount of serum as small as 0.000,006 cc., although *in vitro* reactions of agglutination, precipitation, and complement fixation fail with amounts below 0.002 cc.

2. *The Schwellenwert.*—The maximum amount of culture against which protection can be demonstrated with any amount of serum is termed the *Schwellenwert*. Actually, this threshold is far from exact, since a great many irregular results are obtained. This characteristic is obviously to some extent dependent upon the antibody content of the serum, but there appears to be a final maximum of culture beyond which protection is not obtained in the normal mouse, no matter what the potency of the serum may be. In other words, the *Schwellenwert* is largely determined by the relation between the number of organisms and some host factor or factors. It has been shown that, by increasing the number of cells in the peritoneum, protection can be demonstrated against larger numbers of bacteria. This result suggests that the number of cells may be of considerable significance in fixing the value of the *Schwellenwert*.

3. *The Prozone.*—For each lot of antipneumococcus horse serum there appears to be a particular optimal quantity which gives maximum protection against large numbers of pneumococci. With progressively larger amounts of serum fewer animals survive. This in many respects corresponds to the well known prozone in specific agglutinin and precipitin tests. Contrary to this viewpoint, however, is the fact that antipneumococcus rabbit sera, although possessing antibody titers equivalent to those of horse antisera fail to exhibit this prozone effect. Experiments now in progress indicate that the addition of various heterologous substances may produce an artificial or pseudo-prozone.

The chief purpose of this work has been not only to define the

quantitative characteristics of the protection test *per se*, but also to determine the level of dosage at which maximum variation in the results occurs. In short, if one is to acquire knowledge of the physiological variations or host factors which are so intimately associated with the end-results of any animal test, it is first essential to determine the experimental conditions under which their effects are most evident. In the case of the mouse protection test with antipneumococcus serum, such a fortuitous combination of circumstances occurs in that range of serum and culture relations which gives the most irregular results. It is at this point that inherent physiological differences between various mice, a series of factors which until now has not been controlled, exert their major effect and cause the results in any individual mouse to be unpredictable even though the test be done under so called standard conditions. In subsequent papers an attempt will be made to analyze and evaluate these modifying host factors.

Biological evaluations can be termed quantitatively accurate only if all variables are recognized and controlled. Since the determination of the antibacterial potency of an immune serum is based upon its action in protecting animals against fatal infection, the number of variables is large, and the system, unlike that for the *in vivo* titration of toxin or antitoxin, is in a constant state of flux.

#### SUMMARY

The mouse protection test for the evaluation of type specific antipneumococcus serum has been studied with reference to the quantitative relationships between the amount of antibody and the number of injected bacteria. By varying both these factors through wide limits certain definite characteristics of the protection test have been defined. These are the *Schwellenwert*, the prozone, and the limiting titer zone. The modifications produced in the manifestations of these characteristics by changes in the technic of performing the test and also by certain non-specific reagents are described. The dependence of the outcome of the test upon variable factors intrinsic to the host is discussed.

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# THE PROTECTIVE ACTION OF TYPE I ANTIPNEUMOCOCCUS SERUM IN MICE

## II. THE COURSE OF THE INFECTIOUS PROCESS

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(Received for publication, June 6, 1935)

A preceding paper (1) dealt with the general quantitative aspects of the mouse test for evaluating the protective properties of specific antipneumococcus serum. The present paper is a report of studies concerning the nature of pneumococcus infection in the mouse, and of the modifications of the infectious process induced by the protective action of immune sera.

Although the intraperitoneal infection of mice with pneumococci has long been a standard of laboratory practice, little is known of the nature and course of events in the infective process in these susceptible animals. It was of interest, therefore, to carry out a detailed study covering the following topics: (a) the rate of development of the infection as judged by the multiplication of bacteria; (b) the nature of the cells in the peritoneum and their influence on the course of the infectious process; (c) the changes in these developments induced by the introduction of specific immune serum.

### EXPERIMENTAL

The details of the general experimental method are given in the preceding paper (1). They may be briefly summarized as follows:

*Mice.*—Female mice of the Rockefeller Institute stock were used. The weight range was 17–21 gm.

*Culture.*—Neufeld strain of Type I Pneumococcus. Virulence for mice such that 0.000,000,01 cc. given intraperitoneally invariably produced fatal infection.

*Sera.*—A single lot of Type I antipneumococcus horse serum was used throughout these experiments.<sup>1</sup>

<sup>1</sup> This serum was kindly supplied by Dr. Augustus Wadsworth of the Division of Laboratories of the New York State Department of Health.



*General Method of Infection and Study.*—Mice were injected intraperitoneally with 1 cc. of fluid containing the desired amount of culture and serum, the culture being diluted in broth, the serum in saline. At determined intervals thereafter individual animals were sacrificed by deep ether anesthesia and immediately after death the peritoneal cavity of each animal was thoroughly washed out with 1 cc. of saline. Smears were at once made of these fluids. White cell counts were made by the method commonly used for blood. The smears were stained by the Gram technic and examinations made of a large number of contiguous microscopic fields. The numbers of extracellular and intracellular pneumococci were recorded, and also in the same fields the numbers of white cells showing phagocytosis and those which were negative in this respect were noted. From these data the number of bacteria per cubic millimeter of peritoneal washings was calculated on a proportional basis with reference to the determined number of white cells. It is obvious that information obtained by this method is inaccurate inasmuch as the technic of washing cannot be thoroughly controlled.

Qualitative blood cultures were made by first dipping the tail of the mouse into tincture of iodine, allowing this to dry, and then snipping off the tip with sterile scissors. The blood that exuded was streaked directly on blood agar.

Counts of cells normally present in the peritoneal cavity of control mice of this weight showed considerable individual variation. If, however, one considers the mean count derived from several animals, a fair degree of consistency is obtained.

### *Course of Intraperitoneal Pneumococcus Infection in Mice*

The first experiment was designed to study the course of events following the intraperitoneal injection of virulent pneumococci. No immune serum was administered. Under these circumstances the infected mice die in 12–24 hours. The findings with one experiment of this type are shown in Table I.

Each mouse received 0.1 cc. of Type I pneumococcus broth culture in 1.0 cc. of fluid intraperitoneally. This constitutes more than 10,000,000 minimal fatal infective doses. The method of study was that which has been previously described.

From the data concerning the numbers of cells in the peritoneal cavity, it will be noted that there was no considerable variation until the 2nd hour, at which time a slight increase occurred. At the 4 hour period a marked increase had occurred. Subsequently the number of cells diminished.

A slight decrease in numbers of pneumococci in the peritoneum was noted during the first few minutes but the lag phase of growth was

actually very short. The log phase of growth was demonstrable at the end of the 1st hour, and the increase in numbers was exceedingly rapid up to and including the 4th hour. At the 6th hour the average number of bacteria was somewhat lower, a finding presumably related to the increased numbers of leukocytes present at 4 hours. At 10 hours, however, when the animals were invariably very ill or moribund, the number of bacteria had increased to an exceedingly high figure.

TABLE I

*Course of Intraperitoneal Pneumococcus Infection*

A series of white mice were each injected intraperitoneally with 1.0 cc. of fluid containing 0.1 cc. of 18 hour blood broth culture of Type I *Pneumococcus*. At designated intervals thereafter pairs of mice were sacrificed by abrupt ether anesthesia and the peritoneal cavities immediately washed with 1 cc. of saline. The various determinations were made by methods described in the text. Each of these figures represents the average of determinations on two animals.

| Time elapsing<br>after infective<br>inoculation | White blood<br>cells per<br>c.mm. of<br>peritoneal<br>washings | Calculated<br>extracellular<br>pneumococci<br>per c.mm. of<br>washings | Calculated<br>total<br>pneumococci<br>per c.mm. of<br>washings | White cells<br>showing<br>phagocytosis | Pneumococci<br>intracellular | Phagocytic<br>index |
|---|--|--|--|--|------------------------------|---------------------|
|   |  |  |  | <i>per cent</i>                        | <i>per cent</i>              |                     |
| 1 min.  | 3725   | 15,120   | 15,370   | 1                                      | 1                            | 4                   |
| 15 min.   | 3925   | 11,310   | 11,615   | 2                                      | 3                            | 3                   |
| 30 min.   | 3900   | 11,770   | 13,150   | 7                                      | 10                           | 4                   |
| 1 hr.   | 4050   | 18,940   | 21,051   | 10                                     | 10                           | 5                   |
| 2 hrs.  | 5125   | 44,450   | 51,850   | 18                                     | 13                           | 7                   |
| 4 hrs.  | 22,135   | 193,500  | 220,400  | 16                                     | 10                           | 16                  |
| 6 hrs.  | 7825   | 164,400  | 192,800  | 23                                     | 10                           | 11                  |
| 10 hrs.   | 9570   | 1,114,700  | 1,187,700  | 33                                     | 6                            | 20                  |

All control mice died in 12-24 hours.

Blood cultures showed that pneumococci were invariably present in the blood stream in detectable numbers as early as 15 minutes after intraperitoneal injection.

The figures regarding the per cent of pneumococci intracellular and the per cent of white cells active are possibly very inaccurate, for it is often difficult to determine whether organisms are actually within the cells or merely superimposed. There is no doubt, however, that a considerable degree of phagocytosis does take place even in the absence of specific immune serum. Thus, throughout the course of

this particular experiment an average of almost 10 per cent of all organisms was considered to be within the cells. Since the numbers of bacteria increased with extreme rapidity, more and more cells became active until at 10 hours one-third of all the white cells were participating in the phagocytic reaction. The number of pneumococci taken up by the individual cells was, however, low, averaging only 4 per active cell during the 1st hour, and thereafter approximately 12. These findings are somewhat contrary to the general impression that phagocytosis of virulent pneumococci does not occur in the absence of specific immune serum.

Even though a considerable amount of phagocytosis was demonstrated, there was no evidence that the bacteria were destroyed as a consequence or that the phagocytes were even capable of halting the growth of those bacteria which they had ingested. Microscopic examinations gave ample evidence that the bacteria continued to multiply within the cells and finally brought about their rupture. It is possible, therefore, that the high phagocytic index after the 1st hour may be due to intracellular growth rather than to the actual number of pneumococci phagocytized.

#### *Course of Pneumococcus Infection in Mice Protected by Specific Immune Serum*

In order to learn what changes might be brought about by the administration of immune serum, a series of mice was each given 1 cc. of fluid containing 0.1 cc. of culture together with 0.025 cc. of immune serum, an amount known to protect two out of three mice against fatal infection. It was necessary to use relatively large amounts of culture in order to facilitate the counting of the organisms in the peritoneal fluid. The results of a typical experiment of this order are presented in Table II.

From the data presented in Table II it will be noted that the numbers of white blood cells in the peritoneum varied in much the same manner as did those in mice which received no immune serum. The number of pneumococci had increased at the 30 minute period, but subsequently dropped rapidly. Had the animals received no serum the log phase of bacterial growth would have been apparent at 1 hour. However, in the serum protected mice it was found that at the end of the 1st hour some 67 per cent of the observed pneumococci had been

taken up by phagocytes. The per cent of pneumococci intracellular rose to 100 at the 4 hour period.

In this particular series, somewhat at variance with the findings in other experiments, the numbers of white cells showing phagocytosis in the earlier phases of the infection were not materially different from those at corresponding periods in unprotected mice. However, the number of pneumococci taken up by each cell was much greater in the presence of immune serum. Thus, during the 1st hour in the unprotected mice the average was only 4 per cell, whereas in the animals which received serum the average was 21. This increased efficiency in the

TABLE II

*Course of Intraperitoneal Pneumococcus Infection in Mice Which Had Received Specific Immune Serum*

Each mouse received an intraperitoneal injection of 1 cc. of fluid containing 0.1 cc. 18 hour blood broth culture of Type I Pneumococcus and 0.025 cc. of specific antipneumococcus horse serum.

| Time elapsing<br>after infective<br>inoculation | White blood<br>cells per<br>c.mm. of<br>peritoneal<br>washings | Calculated<br>extracellular<br>pneumococci<br>per c.mm. of<br>washings | Calculated<br>total<br>pneumococci<br>per c.mm. of<br>washings | White cells<br>showing<br>phagocytosis | Pneumococci<br>intracellular | Phagocytic<br>index |
|---|--|--|--|--|------------------------------|---------------------|
|   |  |  |  | per cent                               | per cent                     |                     |
| 1 min.  | 3350   | 18,290   | 19,540   | 4                                      | 7                            | 21                  |
| 15 min.   | 2850   | 16,550   | 19,750   | 2                                      | 16                           |                     |
| 30 min.   | 4150   | 36,450   | 39,200   | 5                                      | 4                            |                     |
| 1 hr.   | 3600   | 2610   | 7930   | 10                                     | 67                           |                     |
| 2 hrs.  | 3850   | 1168   | 4410   | 5                                      | 73                           | 5                   |
| 4 hrs.  | 9850   | 0  | 1667   | 3                                      | 100                          |                     |
| 6 hrs.  | 7400   | 0  | 121  | 1                                      | 100                          |                     |

67 per cent of control mice receiving this combination of serum and culture survived.

latter instance is apparently due to the fact that the pneumococci have been agglutinated by the action of the immune serum, and that clumps of bacteria instead of individual diplococci are engulfed.

In all animals of the control series in which the infection subsequently terminated fatally, pneumococci were demonstrated in the blood as early as 15 minutes after the injection of serum and culture. These findings suggest that the initial reaction in the peritoneal cavity is important in determining the subsequent fate of the animal.

*Comparative Analyses*

By means of this technical procedure the course of pneumococcus infection has been studied under six different conditions, and a comparison will be made of the characteristic findings. However, before enumerating these conditions it will be necessary to define certain terms and procedures.

For each lot of antipneumococcus horse serum there is one definite amount which affords mice the maximum degree of protection against large amounts of culture. This is termed the optimal protective amount of serum. For the particular serum used in these experiments the optimal protective amount was 0.025 cc.

TABLE III

*Survival Rates in Pneumococcus Infections under Various Conditions*

| Nature of material injected   | Normal mice |                 | Mice injected with sodium nucleinate 18 hrs. prior to infection |                 |
|---|-------------|-----------------|---|-----------------|
|   | Group       | Survival rate   | Group   | Survival rate   |
|   |             | <i>per cent</i> |   | <i>per cent</i> |
| 0.1 cc. of Type I pneumococcus broth culture  | A           | 0               | D   | 0               |
| 0.1 cc. of culture plus 0.025 cc. of immune horse serum (optimal protective amount) | B           | 66              | E   | 92              |
| 0.1 cc. of culture plus 0.4 cc. of immune horse serum (prozone amount)              | C           | 5               | F   | 90              |

With amounts of serum greater than this optimum, less protection is obtained. This effect is termed the prozone and an amount of serum chosen to demonstrate this phenomenon is termed a prozone amount. In the following experiments 0.4 cc. of serum was employed.

Certain of the mice in the following experiments had been given an intraperitoneal injection of 0.5 cc. of a 5 per cent solution of sodium nucleinate 18 hours previous to the injection of serum and culture. These animals are hereafter referred to as prepared, indicating that a cellular reaction had been elicited by the previous injection of the irritant. These terms have been defined in detail in a previous paper (1).

The six conditions under which pneumococcus infection of the mouse was studied are listed in Table III together with the survival rates among non-sacrificed controls.

All mice in these series received in the same infective inoculum: 0.1

cc. of Type I pneumococcus broth culture. Group A is made up of mice which received the culture only. Group B comprises those which received an optimal protective amount of antipneumococcus horse serum. Group C includes animals which received a large or prozoning amount of serum. Groups D, E, and F are made up of nucleinate prepared mice similarly treated.

From the survival rates shown in Table III, it will be noted that in the absence of specific immune serum all mice succumbed. With the normal mice the addition of an optimal protective amount of serum led to a survival rate of 66 per cent.

TABLE IV  
*Relative Total White Cells in Peritoneal Fluid*

| Time elapsing<br>after infective<br>inoculation | Normal mice |   |                                 | Prepared mice |   |                                 |
|---|-------------|---|---------------------------------|---------------|---|---------------------------------|
|   | A           | B   | C                               | D             | E   | F                               |
|   | No serum    | Optimal<br>protective<br>amount of<br>serum | Prozoning<br>amount of<br>serum | No serum      | Optimal<br>protective<br>amount of<br>serum | Prozoning<br>amount of<br>serum |
| 1 min.  | 4160*       | 5352  | 5130                            | 8678          | 9290  | 7390                            |
| 15 min.   | 3775        | 3162  | 3400                            | 4540          | 5360  | 7610                            |
| 30 min.   | 5488        | 3308  | 4209                            | 8425          | 6410  | 7590                            |
| 1 hr.   | 3488        | 2777  | 3860                            | 11,888        | 11,905                                      | 8590                            |
| 2 hrs.  | 4950        | 4771  | 2960                            | 9740          | 11,970                                      | 12,340                          |
| 4 hrs.  | 14,020      | 8794  | 5680                            | 20,263        | 11,890                                      | 9486                            |
| 6 hrs.  | 7450        | 8557  | 5540                            | 12,533        | 7903  | 6040                            |
| 10 hrs.   | 9570        | 6089  | 5225                            | 8200          | 4290  | 3350                            |

\* Each figure in this and the following tables represents the mean of determinations on four or more individual animals.

With this amount of serum the survival rate in nucleinate prepared mice was 92 per cent. The use of the prozoning amount of serum in normal mice gave a survival rate of 5 per cent, but this same amount led to the survival of 90 per cent of prepared mice. Although the previous administration of sodium nucleinate had no effect on the survival rate in animals which had received no serum, it markedly enhanced the survival rates in animals which received immune serum.

The data relating to the findings in these six groups will be presented under the following headings: (a) total cells in peritoneal fluids, (b) white cells active as phagocytes, (c) phagocytic indices, and (d) proportions of pneumococci found within cells.

*Total Cells in Peritoneal Fluids.*—The data on the mean numbers of peritoneal cells of mice infected under these several conditions are presented in Table IV.

The following points are worthy of attention in these results.

1. Unprepared mice show the first general increase in numbers of cells at 4 hours, the height of this response being apparently inversely related to the amount of serum injected. Thus the highest mean count in animals which received 0.4 cc. of serum was 5680, in animals which received 0.025 cc. of serum 8794, while in animals which received no serum the peak was 14,020.

TABLE V

*Mean Per Cent of White Cells Active as Phagocytes during Various Periods after Infective Inoculation*

| Group | Character of mice | Immune serum                       | Mean per cent of white cells active as phagocytes |                     |                 |
|-------|-------------------|------------------------------------|---|---------------------|-----------------|
|       |                   |                                    | During 1st hr.                                    | From 2nd to 4th hr. | 6th to 10 hr.   |
|       |                   |                                    | <i>per cent</i>                                   | <i>per cent</i>     | <i>per cent</i> |
| A     | Normal            | No serum                           | 5   | 17                  | 25              |
| B     | "                 | Optimal protective amount of serum | 16  | 11                  | 5               |
| C     | "                 | Prozoning amount of serum          | 5   | 22                  | 40              |
| D     | Prepared          | No serum                           | 7   | 20                  | 43              |
| E     | "                 | Protective                         | 7   | 9                   | 7               |
| F     | "                 | Prozone                            | 4   | 16                  | 4               |

2. In mice prepared by the previous injection of sodium nucleinate the total number of cells was almost twice that found in normal mice. The results with the prepared mice are not as regular as those with the normal animals, but it is apparent that the first general increase in cell count occurred 2 to 3 hours earlier than it did with normal animals.

*White Cells Active as Phagocytes.*—The data are shown in a condensed form in Table V.

It will be noted that in normal mice during the 1st hour the per cent of cells actively phagocytic was highest in those animals which received the so called protective amount of serum. Certain other experimental observations aid in the interpretation of these results.

1. If normal mice are injected with a similar number of rough pneumococci, 30 per cent of the white cells in the peritoneal cavity show phagocytosis at 15 minutes. This finding may be compared to 5 per cent with smooth virulent pneumococci. It would appear that some factor must act as an inhibitor of phagocytosis in the case of the smooth organisms. Most obvious are the capsule and the soluble specific substance derived from it. The specific capsular polysaccharide of Type I *Pneumococcus* was therefore injected in a dilution of 1-100,000 together with rough pneumococci derived from a strain of Type III *Pneumococcus*. Samples of peritoneal fluid taken at 15 minutes showed that on the average only 16 per cent of the cells were actively phagocytic as contrasted to 30 per cent in controls. The mode was much below this figure. These observations suggest that under the conditions of the experiment the specific capsular polysaccharide has the capacity of inhibiting the phagocytic activity of the cells.

2. A second observation which must be taken into consideration in the explanation of these results is the fact that the immune horse serum itself has a similar inhibitory effect. The data presented in Table V show that although with 0.025 cc. of serum 16 per cent of the cells were active during the 1st hour, with 0.4 cc. only 5 per cent showed phagocytosis. Further experiments on the inhibition of phagocytosis have shown that various sera, protein solutions, and lipoids in certain concentrations have a similar inhibiting effect.

In the light of these observations, the reduced phagocytic activity of the cells of mice infected but not receiving immune serum can be explained largely on the inhibitory action of free and fixed capsular polysaccharide. With the addition of an optimal protective quantity of serum, however, this inhibitory influence has been to some extent neutralized. The reduction of phagocytic activity found in mice which received a prozoning amount of serum is explained by the inhibition caused by foreign protein and other substances contained in the serum.

It will be noted from the results shown in Table V that after the 1st hour the phagocytic activity decreased in the group of mice which received optimal protective amounts of serum, but rose sharply in each of the other series. This may be in part explained as follows:

(a) In the mice which received an optimal protective amount of serum the number of extracellular pneumococci became progressively smaller so that under these conditions the number of cells showing phagocytosis is not an accurate index of their potential capacity.

(b) In the other instances, in which phagocytic activity increased sharply, the interpretation is not so obvious. In a later section, however, it will be shown



that there occurs during the course of the infection a shift in the character of the cell picture from one in which the phagocytes are predominantly mononuclear to one in which polymorphonuclear cells are present in great numbers. It may be that the latter cells are not as sensitive as are the mononuclears to the various substances which inhibit phagocytosis. In the instance of the prozone group a further possibility is that some of the serum may have been absorbed, thus lowering the concentration in the peritoneum and thereby lessening the inhibitory effect.

With the nucleinate-prepared mice, the results are somewhat different. It must be recalled that in these animals the total number of cells was originally much higher, and, as will be shown in a later section, many polymorphonuclear cells are initially present. In the group which received no serum the phagocytic activity was somewhat greater than in the corresponding group of normal mice. The proportion of cells active as phagocytes within the serum groups may be explained on the basis of the same factors which are operative in normal mice.

Of particular interest is the fact that the cells of the normal mice show a high rate of phagocytic activity. In spite of this it is known that the injection of a single pneumococcus will bring about death of the animal. Thus, phagocytosis is in itself not an adequate means of defense. It has already been pointed out that without previous specific sensitization the pneumococci tend to multiply within the cell and finally bring about its rupture. It is suggested that immune sera possess the property of rendering the pneumococci sensitive to digestion by the intracellular enzymes of the leukocytes.

*Phagocytic Indices.*—The average number of pneumococci taken up by each active cell is termed the phagocytic index. The results of studies on this point are condensed in Table VI.

It will be noted from the results given in Table VI that during the first 4 hours the actively phagocytic cells of mice which received serum took up significantly greater numbers of pneumococci than did those in mice receiving no serum. Thus during the 1st hour in the normal group there was an average intake of 5 microorganisms per cell when no serum was present. In the protective series the phagocytic index was 18. On the other hand, in the prozone group each active cell took up an average of only 11 pneumococci. Similar results were

obtained in the series of mice prepared by the previous injection of sodium nucleinate.

It is believed that these results may be in part explained on the following basis. The non-inhibited phagocyte apparently takes up particles rather indiscriminately. In serum injected animals these particles consist of agglutinated groups of bacteria rather than of single diplococci. Obviously, in these instances the phagocytosis of clumps of bacteria increases the phagocytic index without indicating an increased activity on the part of the individual cells.

These results show that the phagocytic index is lower in animals which received an excess or prozoning amount of serum than in animals which received an optimal protective amount. Studies with moist

TABLE VI  
*Mean Phagocytic Indices*

| Group                    | During 1st hr. | From 2nd to 4th hr. | From 6th to 10th hr. |
|--------------------------|----------------|---------------------|----------------------|
| A Normal—no serum.....   | 5              | 13                  | 23                   |
| B “ protective.....      | 18             | 20                  | 12                   |
| C “ prozone.....         | 11             | 27                  | 15                   |
| D Prepared—no serum..... | 4              | 10                  | 13                   |
| E “ protective.....      | 15             | 16                  | 11                   |
| F “ prozone.....         | 9              | 19                  | 11                   |

preparations after the method of Etinger-Tulczynska (2) have given a clear explanation of this difference. When a small amount of serum is used the agglutinated mass is extremely compact, the cell bodies proper being in close contact. When, however, large amounts of serum are used, the clumps are made up of cells separated by swollen capsules—that is, these pneumococci show the *Quellung* phenomenon of Neufeld (3). Thus, in a given mass of agglutinated organisms there are fewer bacteria and the efficiency of the system is thereby somewhat diminished.

*Proportions of Pneumococci Found within Cells.*—The end-result in a protection test in terms of the death or survival of the animal is obviously more or less a summation of the combined force of many positive and negative factors. A somewhat more sensitive index,

however, is to be found in the proportion of pneumococci present within cells at various times after infective inoculation. In Table VII are presented the results of studies dealing with the per cent of pneumococci found within cells.

Only in the three groups showing a high survival rate (B, E, F) had all organisms been taken up by cells at 10 hours. In mice with protective amounts of serum, rapidly increasing numbers of bacteria were taken up. In normal mice this reaction had been almost completed at 4 hours, in prepared mice at 1 hour. This difference is not entirely accounted for by the greater numbers of cells in the latter instance, since the initial absolute number of active cells did not differ

TABLE VII

*Pneumococci Intracellular at Various Time Periods*

Figures indicate the mean percentage of observed pneumococci found within cells.

| Group                    | Time after infective inoculation |         |         |       |        |        |        |         |
|--------------------------|----------------------------------|---------|---------|-------|--------|--------|--------|---------|
|                          | 1 min.                           | 15 min. | 30 min. | 1 hr. | 2 hrs. | 4 hrs. | 6 hrs. | 10 hrs. |
| A Normal—no serum.....   | 2                                | 2       | 11      | 8     | 24     | 7      | 16     | 6       |
| B “ protective.....      | 13                               | 15      | 34      | 57    | 66     | 99     | 100    | 100     |
| C “ prozone.....         | 5                                | 3       | 4       | 3     | 7      | 17     | 57     | 14      |
| D Prepared—no serum..... | 28                               | 7       | 9       | 9     | 15     | 28     | 21     | 19      |
| E “ protective.....      | 13                               | 49      | 61      | 99    | 100    | 100    | 100    | 100     |
| F “ prozone.....         | 2                                | 4       | 22      | 25    | 63     | 78     | 97     | 100     |

materially. It may be recalled, however, that the conditioned increase in numbers of cells occurred much earlier in nucleinate prepared mice, and it may be that this was a material factor in bringing about complete phagocytosis. Some explanation of these differences may also be found in the types of cells initially present.

With normal mice which had received a large or prozone amount of serum, the highest per cent of pneumococci intracellular was observed at 6 hours, but the previously unchecked growth of pneumococci undoubtedly presented at this time an insurmountable barrier. With the corresponding group of nucleinate prepared mice, on the other hand, complete phagocytosis had occurred at 10 hours. There is no

readily available explanation for these differences except that of possible differences in types of cells initially present.

*Types of White Cells Found in the Mouse Peritoneum.*—The white cells in the mouse peritoneum have been generally referred to as mononuclears and cannot be differentiated readily in fixed smears. It therefore seemed important to attempt a classification of these cells and a comparison of the cell types of the normal mouse with those in nucleinate prepared animals. These differential studies were carried out by means of the supravital technic of Sabin (4). By this technic the cells usually designated as mononuclears may be easily differentiated into lymphocytes and monocytes. This distinction is important, since the monocytes are potential phagocytes.

TABLE VIII

*Differentiation of the Types of Cells in the Peritoneal Fluids of Normal as Compared with Nucleinate Prepared Mice*

| Mice of same age; weight $18 \pm 0.5$ gm. |                 |                 |
|---|-----------------|-----------------|
| Cell type                                 | Normal mice     | Prepared mice   |
|   | <i>per cent</i> | <i>per cent</i> |
| Polymorphonuclear leukocytes.....         | 0               | 15              |
| Eosinophiles.....                         | 1.5             | 1               |
| Lymphocytes.....                          | 37              | 39.5            |
| Monocytes.....                            | 61.5            | 44.5            |

The monocytes of the peritoneal fluid of the mouse when stained by the supravital method present certain morphological characteristics by which they are easily distinguished from the lymphocytes in the same preparations. The unevenness of the surface films and the irregularity or scalloping of the cellular outline are characteristic of the cells of the monocytic series. The nucleus, which is usually round or oval and rarely indented, is almost invariably centrally situated. Surrounding the nucleus is a zone containing numerous granules and mitochondria, whereas the cytoplasm of the periphery is clear. Considerable variation in the size of these cells has been observed, the smaller ones being no larger than the intermediate lymphocytes in the same preparation, while the largest ones are often three to four times this size. The lymphocytes in the peritoneal fluid closely resemble the lymphocytes of the peripheral blood.

The results of differential studies concerning the types of cells in the peritoneal fluids of normal as compared with nucleinate prepared mice are shown in Table VIII.

From these results it will be noted that polymorphonuclear leukocytes were absent in the peritoneal fluids of normal mice although they constituted 15 per cent of the cells in the case of the prepared mice. Monocytes made up 61.5 per cent of the cells in the peritoneal fluid in normal mice, as compared to 44.5 per cent in prepared animals.

Under these experimental conditions the average total white cells per c. mm. of peritoneal washings of normal mice was 4600, while that of nucleinate prepared mice was 8450. From these figures it is apparent that the injection of the nucleinate brought about an increase in the absolute number of lymphocytes and of monocytes. The most characteristic change, however, is that polymorphonuclear cells, normally absent, have appeared in considerable numbers.

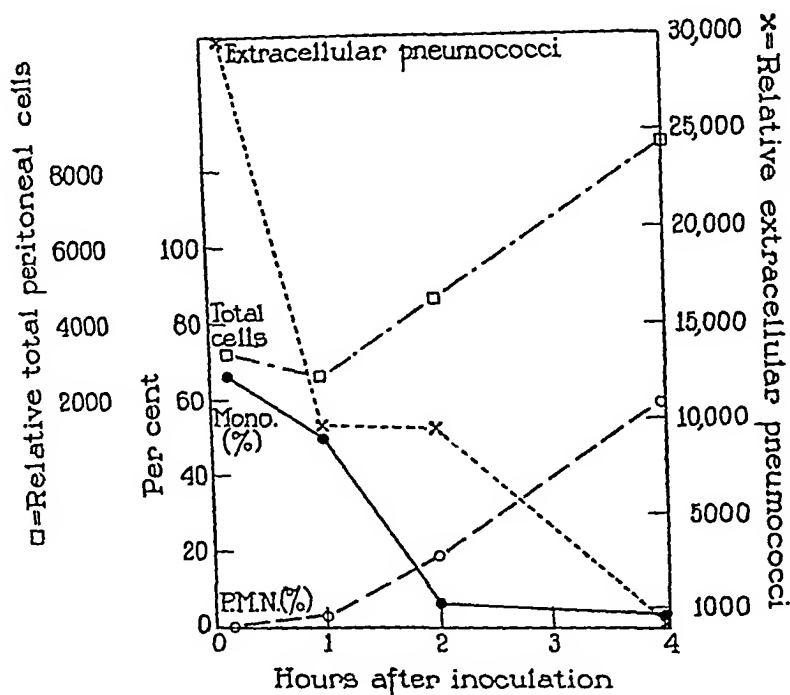
The initial presence of the polymorphonuclear cells undoubtedly contributes to the apparently enhanced protective action obtained with mice which had received a previous injection of sodium nucleinate.

*Cellular Participation in the Protective Process.*—It is of some interest to consider the sequential changes in the proportion of the various cells occurring in the course of the infectious process, and the part which these different types of cells play in the removal of the infectious agents. Supravital differential counts of the cells in the peritoneal fluid have been made at various times after infective inoculation on a group of mice which had received the usual protective amount of serum together with 0.1 cc. of culture. The findings of this study together with certain mean determinations obtained by the previous method are shown in Text-fig. 1.

From these results it will be noted that during the 1st hour the proportion of monocytes fell slightly. After that time, however, there occurred an abrupt drop in the number of these cells. On the other hand, the proportion of polymorphonuclear cells steadily increased from 0 per cent at 15 minutes to 60 per cent at 4 hours. There was at 1 hour a slight decrease in total numbers of white cells. This was followed by a steady increase corresponding roughly to the increase of the polymorphonuclear cells.

The participation of the monocyte in the infectious process, and the reason for the abrupt fall in the number of these cells, are matters of considerable interest. At the 15 minute period it was observed that

some of these cells were already taking up pneumococci. At 1 hour it was observed that marked phagocytosis had occurred, but more significant was the fact that the monocytes were massed in huge clumps about agglutinated pneumococci, and that within these masses the organisms were being rapidly ingested. At 2 hours, however, these masses or large groups of pneumococci and cells were not found. This



TEXT-FIG. 1. Composite chart showing the changes in the proportions of cell types in the peritoneal fluid after injection of serum and culture, correlated with the number of extracellular pneumococci at various times.

disappearance coincided with the disappearance of the monocytes from the peritoneal fluid. It is therefore rationally presumed that the massing of these cells had formed particles too large to remain suspended in the peritoneal fluid. It would appear that the observed decrease in the proportion of monocytes gives a false impression, and that these cells probably continue active in the destruction and elimination of the bacteria.

With the appearance of large numbers of polymorphonuclear cells, marked phagocytosis was again observed.

It is of some interest to note that the number of extracellular pneumococci had dropped abruptly during the 1st hour. During the 2nd hour, when the proportion of monocytes in the fluid was apparently decreasing and the number of polymorphonuclear cells had not yet reached any considerable level, there was little appreciable change in the number of free pneumococci. This probably represents a balance between two processes, phagocytosis on the one hand and bacterial growth on the other. From the 2nd to the 4th hour there apparently occurred a second wave of elimination of free pneumococci, this being associated with the inrush of polymorphonuclear cells.

#### DISCUSSION

An attempt has been made to study the sequence of events following the intraperitoneal injection of mice with pneumococci and to learn in what manner this sequence might be altered by the administration of immune serum. As in any infectious process of this order, the number of forces or factors involved in the production of the end-result is very large. An infection represents a constant state of flux, and the tendency in either direction toward a state of equilibrium is continuously influenced by numerous opposing forces often confusingly interrelated. The complexity of this succession of events is so great as to forbid any complete simulation by *in vitro* methods. Infection can only be studied by direct observation, and the results interpreted in the light of isolated and carefully controlled experiments. The data presented in this study, although inadequate, have permitted the figurative reconstruction of certain phases of the infectious process and a rough evaluation of some of the factors which influence its course.

The number and nature of the cells present in the peritoneum at the time of injection of serum and culture have been shown to have an important bearing on the subsequent infectious process. These studies point to the importance of the monocyte in the defense reaction of the host. This cell appears to form the first line of defense of the tissues, for it is the actively phagocytic element normally present and

serves to hold the infectious agent in check until the polymorphonuclear cells of the blood have responded to the stimulus.

The present studies support the view that antipneumococcus serum owes its protective properties to three specific actions: (a) the neutralization of the capsular polysaccharide and the consequent elimination of its inhibition of phagocytosis; (b) the agglutination of bacteria, permitting a greater efficiency in the phagocytic action in that clumps of bacteria rather than single diplococci may be engulfed; and (c) the sensitization of the pneumococci which favors subsequent intracellular enzymatic digestion, a process to which virulent organisms are resistant in the unsensitized state.

#### SUMMARY

Observations are reported which concern the nature of the infectious process resulting from the intraperitoneal injection of mice with virulent pneumococci.

The course of the infection has been figuratively reconstructed on the basis of the following data: The rate of bacterial multiplication, the numbers of cells present in the peritoneal cavity, the character of these cells at various stages, and the rate of phagocytosis.

The significant alterations in this infectious process brought about by the administration of type specific immune serum are described, and the general significance of the findings discussed with reference to the functions of the immune serum and the rôle of phagocytes in protection.

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# THE PROTECTIVE ACTION OF TYPE I ANTIPNEUMOCOCCUS SERUM IN MICE

## III. THE SIGNIFICANCE OF CERTAIN HOST FACTORS

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Type specific antipneumococcus sera have, in general, the capacity to protect mice against infection with virulent organisms of the homologous type. The quantitative limitations of the protection test in mice have been pointed out in the first paper of this series (1), in which attention was also called to the many irregularities in the results. In a wide range of graded doses of serum and culture some mice of each group die whereas others survive. The irregular and unpredictable fatalities that occur under standard test conditions are attributed to variations among animals in their capacities to utilize the available protective elements of the immune serum.

A previous study (2) has dealt with this problem in pneumococcus infections in rabbits. In this instance it was found that heavier animals and those having high white blood cell counts at the time of infective inoculation were much better able to utilize the passively conferred immune principles. No evidence was obtained, however, to show that either the weight or the number of cells was other than a reflection of some more complex physiological system.

The present paper is essentially an extension of this problem as applied to pneumococcus infection in the white mouse and concerns the interrelations of the amount of immune serum, the number of infecting organisms, and certain physiological variables or individual host factors, all of which play important rôles in determining the outcome of a protection test in any given animal.

## EXPERIMENTAL

The general method employed was the same as that described in a previous paper (1).

*Mice.*—Females of the Rockefeller Institute strain of white mice were used throughout.

*Culture.*—Pneumococcus Type I, original Neufeld strain. Virulence for mice such that 0.000,000,01 cc. of broth culture given intraperitoneally invariably produced fatal infection.

*Sera.*—Antipneumococcus horse serum, Type I, was used.<sup>1</sup>

Serum and culture were diluted so that the desired amounts of each were contained in a volume of 0.5 cc. Equal parts of each were then mixed in a syringe and injected intraperitoneally.

### *General Method of Approach*

Previous studies (1, 3) have shown that if prior to infection the number of cells in the peritoneal cavity are increased by non-specific stimulation the animal is much more apt to be protected by a given quantity of serum. The beneficial effect of the cellular response was so striking that a method was sought by which the number of cells in the peritoneal fluid might be estimated. It was most desirable to have this estimation made at about the time serum, bacteria, and body cells came together, and certainly before the initiation of the log phase of bacterial growth. To obtain an estimate before infection would require the injection of some sort of microscopic tags. An examination of the previous data suggested the use of the pneumococci themselves as the tags. This is practicable in this instance since it has been shown that the lag phase of growth persists for at least 30 minutes; that in serum protected animals there is no apparent diminution in numbers of bacteria during this time; and finally that the numbers of cells do not vary materially during this period. If then a small quantity of the peritoneal fluid be removed during this period one should be able to determine by examination of smears the number of white cells in relation to the number of pneumococci. The actual procedure as finally established was as follows:

*Mice.*—In each series of mice the weights were recorded.

*Injection.*—The culture was diluted in broth so that the desired amount was contained in 0.5 cc. volume. A similar system of dilution in saline was used for the immune serum. Culture and serum were mixed in the syringe before injection.

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<sup>1</sup> This serum was furnished through the courtesy of Dr. Augustus Wadsworth of the Division of Laboratories of the New York State Department of Health.

tion. The intraperitoneal injection was made just lateral to the left caudal mammary gland. The time of injection was carefully noted.

*Sampling.*—Sterile capillary pipettes were used to obtain the necessary drop of fluid. The actual sampling is accomplished by a quick thrust of the pipette on the side opposite to that of injection, and this is followed by a slow withdrawal. Sampling was carried out just 15 minutes after the injection of serum and culture. Preliminary experiments showed that sampling before 5 minutes was unsatisfactory because thorough mixing of the injected matter with the peritoneal contents had not occurred.

*Smears.*—The fluid obtained was spread rather thickly on glass slides and stained by the Gram technic. The numbers of cells and bacteria in successive contiguous microscopic fields were accurately and carefully counted. A minimum of 1000 pneumococci or of 500 white cells were counted in each preparation. In case any specimen showed a very irregular distribution as, for example, massive clumping of bacteria or white cells, this minimal requirement was doubled. A separate record was made of cells showing phagocytosis and of those that contained no organisms. Likewise the number of intracellular and extracellular pneumococci were noted. It must be emphasized that consistent results can be obtained by this method only by the most painstaking and meticulous examination of the smears.

An example of the results of a single experiment of this type is shown in Table I.

This experiment included a series of eight mice of the same weight (17.5 gm.). Each animal received an intraperitoneal injection of 0.05 cc. of specific anti-pneumococcus horse serum and 0.1 cc. of Type I pneumococcus broth culture. Samples of peritoneal fluid were taken 15 minutes after infective inoculation. For each smear the separate determinations are recorded and the total cells and total pneumococci in the same fields calculated. From these data is calculated the ratio of total pneumococci to total cells. It will be noted that these ratios range from 3.7–14.6. Since each mouse received the same amount of culture these ratios obviously are inversely representative of the number of cells in the peritoneal cavity. Thus Mouse 6, which showed 3.7 pneumococci per cell, obviously had many times more cells than Mouse 7, which showed 14.6 pneumococci per cell. By a process of proportional calculation it is possible to arrive, as originally planned, at a numerical estimation of the total numbers of cells in the peritoneal cavity of each animal. However, as will be shown later, the total number of cells is without significance except as related to the total number of bacteria. Moreover, the technical difficulties inherent to the injection of an exact number of pneumococci tend to lessen the accuracy of the total cell estimate.

An examination of the results presented in the last column of Table I shows that only two of the eight mice receiving 0.025 cc. of serum

and 0.1 cc. of culture succumbed to infection. These two were the animals with the highest pneumococcus/cell ratios; that is, those with the fewest peritoneal cells. Moreover, there is a sharp limiting threshold, for although Mouse 4, with a ratio of 10.1, died, Mouse 2, with a ratio of 9.8, survived.

Our experience with this type of study has now covered more than 1500 animals and irregular results do not exceed 15 per cent. In our

TABLE I

*Type Protocol of Mouse Protection Test*

Weight range:  $17.5 \pm 0.3$  gm. Each mouse in the following series received an intraperitoneal injection of 1 cc. of fluid containing 0.05 cc. of antipneumococcus horse serum (Lot E) together with 0.1 cc. of an 18 hour blood broth culture of Type I Pneumococcus. 15 minutes after injection samples of peritoneal contents were obtained and from these smears prepared. After the application of the Gram stain large numbers of contiguous microscopic fields were traversed and the following numbers of cells and pneumococci observed.

| Mouse No. | Observed cells |          |       | Observed pneumococci |                |       | Total pneumococci<br>Total cells | Result |
|-----------|----------------|----------|-------|----------------------|----------------|-------|----------------------------------|--------|
|           | Active         | Inactive | Total | Intra-cellular       | Extra-cellular | Total |                                  |        |
| 1         | 25             | 395      | 420   | 530                  | 1103           | 1623  | 3.9                              | S      |
| 2         | 10             | 132      | 142   | 178                  | 1265           | 1443  | 9.8                              | S      |
| 3         | 0              | 148      | 148   | 0                    | 1018           | 1018  | 6.9                              | S      |
| 4         | 17             | 196      | 213   | 468                  | 1679           | 2147  | 10.1                             | D 80   |
| 5         | 23             | 254      | 277   | 372                  | 1439           | 1811  | 6.5                              | S      |
| 6         | 0              | 270      | 270   | 0                    | 1001           | 1001  | 3.7                              | S      |
| 7         | 7              | 84       | 91    | 210                  | 1115           | 1325  | 14.6                             | D 45   |
| 8         | 17             | 122      | 139   | 488                  | 711            | 1199  | 8.6                              | S      |

S = survival.

D = death within the indicated number of hours after infective inoculation.

hands the results attained by this technic have served as a basis for prediction of death or survival in the majority of all animals in any given series.

*Variations in the Amount of Culture*

This type of result suggests that the absolute number of cells may be important only as related to the number of pneumococci. If this is true the significance of the ratios should be maintained irrespective

TABLE II

*The Initial Pneumococcus/Cell Ratios in Relation to the Protective Action of Anti-pneumococcus Serum*

Weight range: 22-24.5 gm.

Each of the following mice received an intraperitoneal injection of 0.1 cc. anti-pneumococcus horse serum together with the indicated amount of Type I Pneumococcus culture.

The figures given below express the ratio

$$\frac{\text{Total observed pneumococci}}{\text{Total observed cells}}$$

| Amount of culture |         |         |
|-------------------|---------|---------|
| 0.05 cc.          | 0.1 cc. | 0.2 cc. |
|                   |         | 13.0 D  |
|                   | 11.9 D  | 11.0 D  |
|                   |         | 10.3 D  |
|                   | 8.0 D   |         |
|                   | 8.0 D   |         |
|                   |         | 7.2 D   |
|                   |         | 6.1 D   |
|                   | 6.0 D   | 6.1 D   |
|                   | 5.5 D   | 5.6 D   |
|                   | 5.5 D   |         |
| 5.3 D             | 5.4 D   | 5.0 D   |
|                   | 4.9 D   |         |
| 4.4 D             | 4.2 D   | 4.5 D   |
| 4.1 D             | 4.1 D   |         |
|                   | 4.1 S   |         |
|                   | 4.0 D   |         |
|                   | 4.0 S   |         |
|                   | 3.3 S   |         |
|                   | 3.2 S   |         |
| 2.9 S             |         |         |
| 2.5 S             |         |         |
| 1.5 S             | 1.8 S   | 2.7 S   |
| 1.2 S             | 1.6 S   |         |
| 1.1 S             | 1.3 S   |         |
| 0.7 S             |         |         |
| 0.3 S             | 0.1 S   |         |

S = survival.

D = death within 6 days.

of the amount of culture injected. In order to test this hypothesis a series of mice of comparable weights were injected with an infective inoculum containing the same amounts of immune serum but with different amounts of culture. The results of this experiment are shown in Table II.

It will be noted in Table II that in the series of 19 mice which received 0.1 cc. of culture the limiting ratio was 4.0–4.1; that is, all mice showing smaller ratios survived, whereas those with ratios higher than 4.1 died. Similarly in the series of ten mice which received 0.2 cc. of culture the limiting ratio was between 2.7 and 4.5. In the series which received 0.05 cc. of culture the limit was between 2.9 and 5.3. Unfortunately in these latter instances the critical threshold values are not as sharply defined as in the preceding series. However, it is quite evident that the limiting ratios are of the same order of magnitude irrespective of the number of pneumococci injected.

These results lend support to the view that the critical variable is not the absolute number of cells alone, but is rather the number of cells in relation to the number of pneumococci.

#### *The Threshold and the Type of Cells*

In the second paper of this series (3) it was pointed out that normal mice under the experimental conditions described have few or no polymorphonuclear cells in the peritoneal cavity except after irritation or stimulation. From studies (1) dealing with mice which had received a previous injection of sodium nucleinate it was inferred that the protection was improved by virtue of the presence of polymorphonuclear cells. If this were true the effective ratios should be higher in nucleinate prepared mice than in normal mice; that is, fewer total cells should be more effective because of the greater proportion of phagocytes. In order to test this hypothesis a series of mice was divided and half given intraperitoneal injections of sodium nucleinate 18 hours prior to the injection of serum and culture. In order to obtain comparable pneumococcus/cell ratios it was necessary to inject the prepared mice with 0.4 cc. of culture as opposed to 0.1 cc. with normal mice. All received the same amounts of immune serum. The results of this experiment are shown in Table III.

It will be noted that with normal mice the threshold ratio was

between 4.4 and 7.2. On the other hand in the series of prepared mice the critical value was between 10.5 and 13.0. In other words with the prepared mice, the proportion of phagocytic cells being greater, fewer

TABLE III

*Critical Pneumococcus/Cell Ratios in Normal Mice as Compared to Mice Which Had Received a Previous Injection of Sodium Nucleinate*

Weight range 21-22.5 gm.

In the following table the term nucleinate prepared refers to the fact that each of these mice had received an intraperitoneal injection of 0.5 cc. of a 5 per cent solution of sodium nucleinate 18 hours prior to infection.

| Mouse No. | Character           | Serum | Culture | Total observed pneumococci<br>Total observed cells | Result |
|-----------|---------------------|-------|---------|--|--------|
|           |                     | cc.   | cc.     |  |        |
| 1         | Normal              | 0.05  | 0.1     | 8.1  | D 23   |
| 2         | "                   | 0.05  | 0.1     | 7.2  | D 23   |
| 3         | "                   | 0.05  | 0.1     | 4.4  | S      |
| 4         | "                   | 0.05  | 0.1     | 3.5  | S      |
| 5         | "                   | 0.05  | 0.1     | 2.9  | S      |
| 6         | "                   | 0.05  | 0.1     | 2.1  | S      |
| 7         | "                   | 0.05  | 0.1     | 1.5  | S      |
| 8         | "                   | 0.05  | 0.1     | 0.6  | S      |
| 9         | Nucleinate prepared | 0.05  | 0.4     | 19.3   | D 95   |
| 10        | " "                 | 0.05  | 0.4     | 13.0   | D 95   |
| 11        | " "                 | 0.05  | 0.4     | 10.5   | S      |
| 12        | " "                 | 0.05  | 0.4     | 7.4  | S      |
| 13        | " "                 | 0.05  | 0.4     | 7.4  | S      |
| 14        | " "                 | 0.05  | 0.4     | 5.9  | S      |
| 15        | " "                 | 0.05  | 0.4     | 5.0  | S      |
| 16        | " "                 | 0.05  | 0.4     | 3.0  | S      |

S = survival.

D = death within the indicated number of hours after infective inoculation.

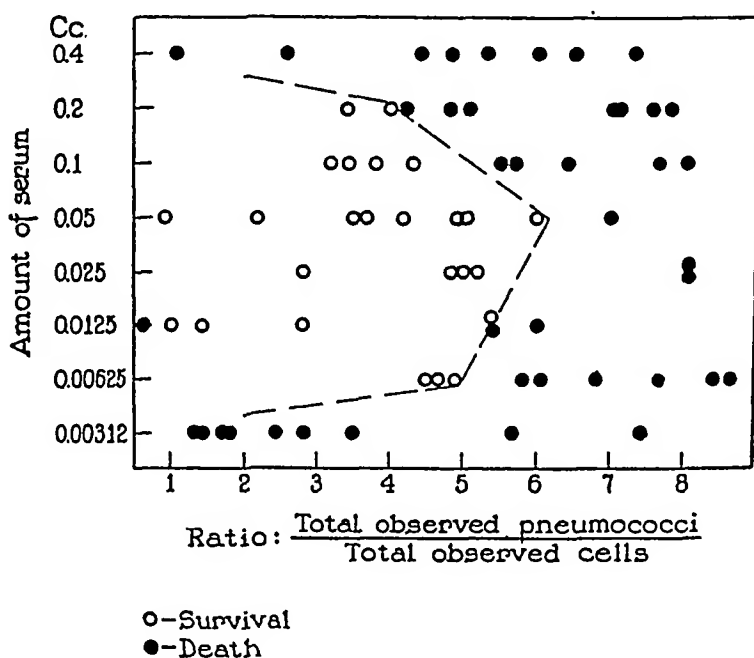
total cells could accomplish the removal of the bacteria than with mice in which polymorphonuclear cells are initially absent.

#### *Variations in Amount of Serum*

It has been shown in a previous paper (1) that there appears to be an optimal amount of serum which should be used in order to confer protection on the greatest number of animals. If smaller or larger amounts are employed the percentage survival is lower. It has been



shown that with the larger amounts of serum there is an inhibition of the phagocytic mechanism, that with the smaller amounts, the organisms appear to have been insufficiently sensitized so that growth takes place within the cell in spite of phagocytosis (3). For these reasons the amount of serum should modify the significance of the pneumococcus cell ratio. In order to test this hypothesis a series of mice of comparable weights were each given the same amount of culture but varying amount of immune serum. A typical example of this type of experiment is shown in Text-fig. 1.



TEXT-FIG. 1. Critical pneumococcus/cell ratios with varying amounts of immune serum. Each mouse received 0.1 cc. of the standard culture.

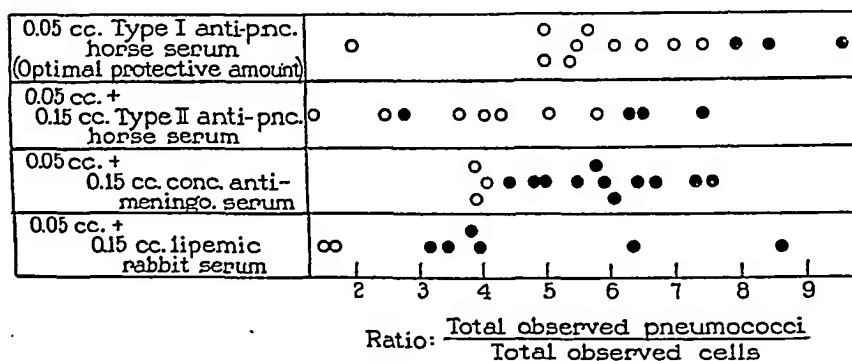
In this series the general weight range was 18–23 gm. although for each subgroup the weight variation was not over  $\pm 0.5$  gm. Each mouse received 0.1 cc. of an 18 hour blood broth culture of Type I pneumococcus together with the indicated amount of serum. The standard technic was used throughout.

The optimal protective amount of this particular lot of immune serum was 0.05 cc. It will be noted from Text-fig. 1 that at this level the limiting ratio was clearly between 6.0 and 7.0. With 0.1 cc. of serum the threshold lay between 4.3 and 5.5; with 0.2 cc. between 4.0

and 4.2; while with 0.4 cc. of serum the limiting value was obviously below 1.1. Similarly with smaller amounts of serum the limiting ratio became lower as less serum was used.

Thus, although the limiting or threshold ratio appears not to be affected by the amount of culture, it varies decidedly with the amount of immune serum.

The effective ratio can also be greatly modified by the addition of heterologous materials. In Text-fig. 2 are shown the results obtained by the addition of comparable amounts of Type II antipneumococcus serum, concentrated antimeningococcus serum, and lipemic rabbit serum.



o—Survival

●—Death

TEXT-FIG. 2. Critical pneumococcus/cell ratios as affected by various heterologous sera. Each mouse received 0.1 cc. of the standard culture.

In this experiment mice weighing 17.5–19.5 gm. were used. Each mouse received 0.05 cc. of Type I antipneumococcus horse serum, 0.1 cc. of culture, and in certain instances 0.15 cc. of the indicated heterologous serum. The standard technic was used throughout.

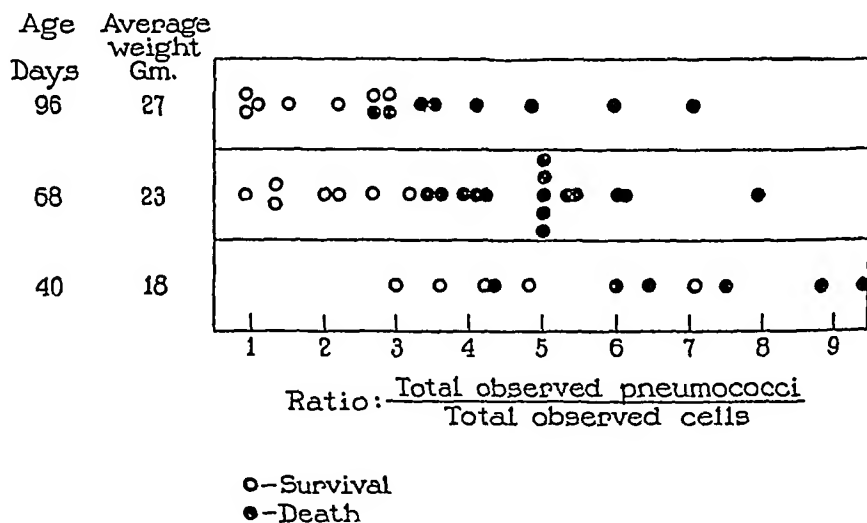
It will be noted that the threshold ratio in the control series was between 7.4 and 7.9. With the addition of Type II antipneumococcus serum the threshold was lowered to between 5.8 and 6.3, with concentrated antimeningococcus serum to between 4.1 and 4.4, while with lipemic rabbit serum the limiting ratio fell to between 1.7 and 3.2.

These experiments tend to support the theory that the limiting

effective ratio can be greatly altered by means of non-specific agents as well as by the amount of specific immune serum.

*The Influence of the Age and Weight of the Animal*

The previous experiments, in which mice of comparable weights were used, have clearly shown the importance of the pneumococcus/cell ratio. Since this ratio reflects the total number of cells, the results indicate that the latter is a significant host factor. It was early recognized that without weight control the experimental results showed little consistency. Although this and previous experiences



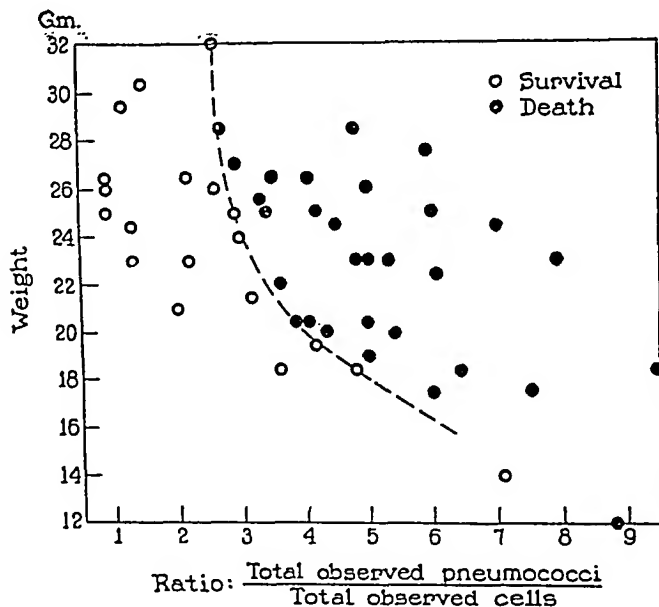
TEXT-FIG. 3. Critical pneumococcus/cell ratios in mice of different ages. Each mouse received identical amounts of serum and culture.

suggested that body weight might be a significant host factor there remained the possibility that the actual factor was age instead of weight. In order to test this possibility a large number of mice in three different ages were each injected with exactly the same amounts of serum and culture and the pneumococcus/cell ratios determined in usual fashion by the method described.

Each mouse received 0.1 cc. of Type I antipneumococcus serum (Lot E) together with 0.1 cc. of the standard Type I pneumococcus culture. The mice were of three ages; 96, 68, and 40 days. Their mean weights for each group were 27, 23, and 18 gm. respectively. The results of this experiment are shown in Text-fig. 3.

From these results, graphically presented in Text-fig. 3, it will be noted that in the oldest age group the limiting ratio was *ca.* 2.6–2.9, with the intermediate group *ca.* 3.3, while with the youngest mice in the series no sharp end-point was obtained.

In general it would appear from these results that with increasing age the limiting ratio becomes smaller. Since, however, the end-points in the age groups were not entirely distinct the same data were re-



TEXT-FIG. 4. Critical pneumococcus/cell ratios in mice of various weights. Each mouse received identical amounts of serum and culture.

plotted against weight rather than age. The results of this replotting are shown in Text-fig. 4.

In Text-fig. 4 a broken line has been inserted to indicate the suggested boundary between zones of protection and non-protection. This boundary is essentially that of the limiting ratio at various weights under these conditions. It will be noticed that the limiting ratio becomes lower as the weight increases. It would appear that a given number of cells is more effective in the protection process in small mice than in large mice.

In seeking an explanation for these results it was recalled that in a previous study (3) the importance of the monocyte had been most strikingly demonstrated. It was therefore determined to examine mice of various weights to learn if the proportion of monocytes varies with the weight of the animal. The result of this study, carried out by means of the supravital technic of Sabin (4), are shown in Table IV.

From these data it will be noted that in the weight range of 12–15 gm. monocytes made up 77.5 per cent of the cells in the peritoneum, whereas in mice weighing 29–33 gm. the proportion of monocytes was only 19.9 per cent. These results indicate that the proportion of monocytes in the peritoneum is roughly inverse to the weight of the animal.

TABLE IV

*Differential Analysis of the Cells Normally Present in the Peritoneal Cavity of the White Mouse*

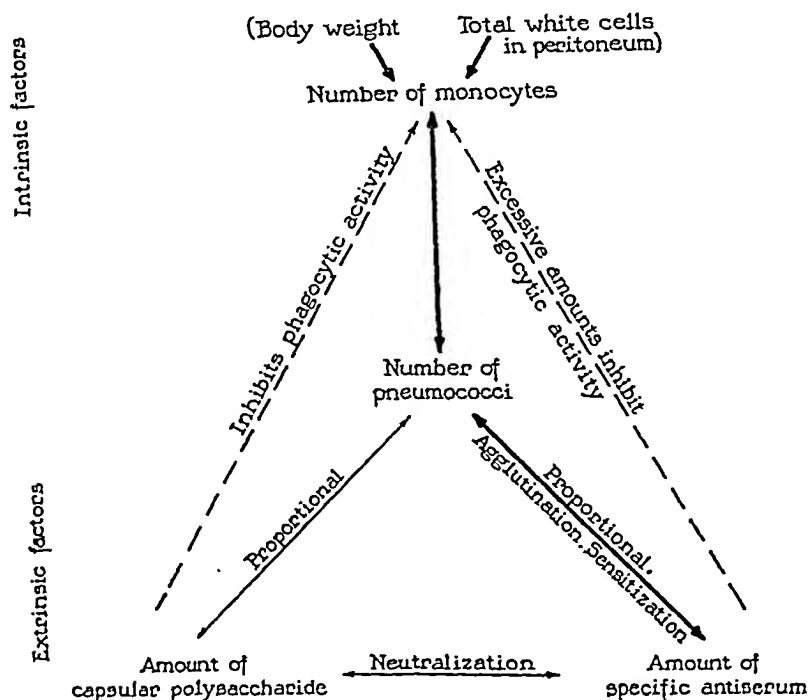
| Weight group<br>gm. | Number of mice | Cell differentials     |                 |                 |                 |
|---------------------|----------------|------------------------|-----------------|-----------------|-----------------|
|                     |                | Polymorpho-<br>nuclear | Eosinophiles    | Lymphocytes     | Monocytes       |
|                     |                | <i>per cent</i>        | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> |
| 12–15               | 6              | 0                      | 0.7             | 21.8            | 77.5            |
| 15.5–18             | 12             | 0.2                    | 0.8             | 38.2            | 60.8            |
| 18.5–21             | 13             | 0.6                    | 0.9             | 50.3            | 48.2            |
| 21.5–24             | 13             | 0.2                    | 0.1             | 57.9            | 41.8            |
| 24.5–28.5           | 7              | 0                      | 0.5             | 64.0            | 35.5            |
| 29–33               | 6              | 1.2                    | 0.7             | 78.2            | 19.9            |

The significance of this finding is at once obvious since it has been shown that the critical pneumococcus/cell ratios also vary inversely with the weight of the animal. Thus there is a direct parallelism between the initial proportion of monocytes in the peritoneum and the effectiveness of the protective system. This relationship is not surprising in view of the fact that the monocyte is the only phagocytic cell initially present in the peritoneum.

Although technical difficulties have prevented a direct demonstration, it is possible, on the basis of these correlated facts, to resolve both the weight and the total number of cells into one host factor, the absolute number of monocytes.

## DISCUSSION

This study has dealt with certain physiological variables of the individual mouse as related to its capacity to utilize the protective qualities of antipneumococcus serum. The problem was to some extent oriented by findings in a previous study dealing with pneumococcus infection in the rabbit (2). As in this earlier work it was found



TEXT-FIG. 5. Initial interrelations of extrinsic and intrinsic variables in the mouse protection test.

that in mice the weight of the animal and the number of white cells at the site of infection serve as quantitative indices of the animal's capacity to be protected. These two individual host factors were resolved in theory into a single correlated component; *viz.*, the number of monocytes present in the peritoneum at the time of injection of serum and culture.

From several points of view this finding is not illogical. The

monocyte is the only actively phagocytic cell normally present in the peritoneal cavity of the white mouse. In a previous paper (3) it was shown that polymorphonuclear elements do not mobilize in significant numbers short of 2 hours after the injection of foreign material. Similarly it was demonstrated that if unchecked during this period the number of bacteria increases by a multiple of four. Thus the monocyte is the cell which must bear the larger part of the defensive burden during this initial period. Hence the number of these cells available is exceedingly important and indeed conditions the entire subsequent course of the infection.

In so far as can be determined now the relationships of the initial variables concerned in the mouse protection test as carried out in these experiments may be illustrated in Text-fig. 5. The extrinsic factors are the physiological variables: body weight and number of cells in the peritoneal cavity. These are resolved into the number of monocytes, which in turn is related to each of the three extrinsic components of the system; *viz.*, the amount of immune serum, the number of pneumococci, and the amount of capsular polysaccharide. By far the most dominant relationship is between the number of monocytes and the number of pneumococci but the activity of the cells may be greatly affected by excessive amounts of serum and by the amount of free capsular polysaccharide. The immune serum serves three useful functions: the agglutination of the pneumococci; the sensitization of these organisms to the digestive action of the cellular enzymes; and neutralization of the free capsular polysaccharide which is injected with the pneumococci. There is no intent in this schematic representation to weigh all of the factors involved in the infectious process but merely to present those which appear dominant in the earliest and most critical period.

#### SUMMARY

The power of specific antipneumococcus serum to protect mice against infection with Type I Pneumococcus has been studied with reference to the capacity of the animal to utilize the specific antibodies. With a single strain of mice it was found that smaller animals and those with large numbers of white cells in the peritoneal cavity are much better able to utilize the passively conferred immune principles.

These two intrinsic factors were resolved into a single element; namely, the number of monocytes in the peritoneal cavity at the time of injection of culture and serum. The interrelation of the extrinsic and intrinsic factors participating in the process of protection have been discussed.

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# AN APPARATUS FOR THE CULTURE OF WHOLE ORGANS

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PLATE 15

(Received for publication, June 12, 1935)

The apparatus described in this paper was designed to maintain a sterile, pulsating circulation of fluid through living organs. More than twenty-six experiments, with various organs, have been made up to the time of writing. Some of the results have already been reported in brief.<sup>1</sup>

The apparatus may be considered to consist of two portions. One, the perfusion pump, contains the organ and perfusion fluid, and must be handled with aseptic technique. The other is for the purpose of creating and transmitting a pulsating gas pressure to the perfusion fluid contained in the first portion. It is unnecessary to keep the second portion sterile. The perfusion pump is made of pyrex glass,<sup>2</sup> and has only three openings which communicate with the exterior. These openings are protected against infection by filter bulbs containing non-absorbent cotton. Neither the organ nor the perfusion fluid comes in contact with any corks or joints which communicate with the exterior. The perfusion pump involves the use of three glass chambers, one above another. The organ lies on the slanting glass floor of the highest chamber. Fluid from the lowest, or reservoir, chamber is driven through the nutrient artery by pulsatile gas pressure. After passing through the organ, the fluid returns through the central chamber, back to the reservoir. The central chamber exists for pressure equalization.

The apparatus is actuated by compressed air controlled by a rotating valve. The pulsations created are transmitted through an oil

<sup>1</sup> Carrel, A., and Lindbergh, C. A., *Science*, 1935, 81, 621.

<sup>2</sup> All glass contained in the apparatus was blown by O. Hopf of The Rockefeller Institute.

column to controlled gas which passes back and forth through the cotton filter bulbs and causes the perfusion fluid to circulate.

The composition of all gas in contact with the organ and the perfusion fluid is controlled. Filming and evaporation of the fluid are prevented. The maximum and minimum pulsation pressures and the pulsation rate are adjustable. The pressure at various points in the pulse cycle can be controlled. The temperature of operation is adjustable. The rate of flow of perfusion fluid can be measured. Changes in rate of flow through the organ are adjusted for automatically with a minimum effect on pulsation pressures. The perfusion fluid is filtered during its circulation and before it enters the organ. Organs can be removed from one apparatus and installed in another aseptically. The perfusion fluid can be removed and replaced aseptically. The organ and the perfusion fluid can be observed at all times.

### *Principle of Circulation and Pulsation*

The basic principle of the pulsating circulation is built upon the use of three chambers placed in vertical line. The lower chamber (*R*) is the fluid reservoir. The upper chamber (*O*) is the organ chamber, and the center chamber (*E*) is the pressure equalizing chamber.

The pulsation and circulation in the apparatus is actuated by a pulsating gas pressure in the gas line (*G*). For the purpose of this explanation the pressure in the gas line (*G*) is set to vary from approximately 0 mm. Hg to 120 mm.

The gas line (*G*) branches just before entering the reservoir and equalization chambers (*R* and *E*). One branch leads directly to the equalization chamber (*E*) and transmits its pressure (approximately 0 mm. to 120 mm.) to the chamber (*E*). The other branch divides again and carries the pulsating gas through a one-way valve (*V*) in one line and a constricted opening (*C*) in the other. Then the two divisions come together again into a single line which enters the reservoir chamber (*R*). The one-way valve (*V*) offers no resistance to gas entering the reservoir chamber (*R*) and consequently, the maximum pressure of the gas line (120 mm.) is exerted directly on the reservoir chamber (*R*). The constricted opening (*C*) bypassing the one-way valve (*V*) is, for this explanation, so adjusted that only enough gas can leak back to reduce the pressure in the reservoir chamber (*R*) to 60 mm. Hg before the next pulsation brings it again to 120 mm.

By varying the maximum pressure in the gas tube line (*G*), and the size of the constricted opening (*C*), bypassing the one-way valve (*V*), the maximum and minimum pressures exerted on the organ can be set at any amount desired.

For the purpose of explanation of the operating principle, the organ chamber (*O*) may be considered as always under a pressure of approximately 0 mm. Hg. The reservoir chamber (*R*) has been adjusted to a pulsating pressure varying from 60 mm. minimum to 120 mm. maximum. Consequently, the equalization chamber (*E*) is under 120 mm. pressure when the reservoir chamber (*R*) is under 120 mm.; and is under approximately 0 mm. when the reservoir chamber (*R*) is

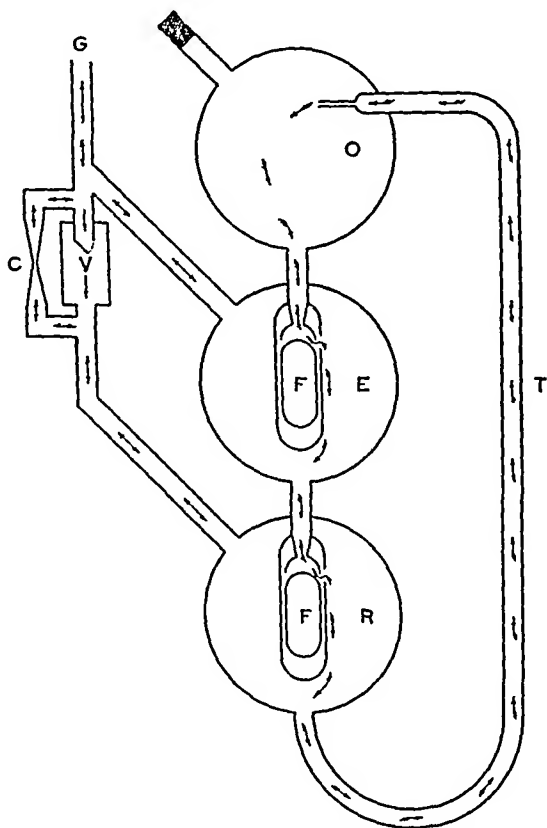


CHART 1. Working diagram of pulsating perfusion pump.

under 60 mm. In other words, during one portion of each pulsation the pressure in the equalization chamber (*E*) equals the pressure in the reservoir chamber (*R*). During another portion of the pulsation the pressure in the equalization chamber (*E*) equals the pressure in the organ chamber (*O*). The artery of the perfused organ is attached to a cannula leading from the reservoir chamber (*R*), and consequently is always approximately under the pressure of the reservoir chamber (60 mm. to 120 mm. Hg).

After the perfusion fluid passes through the organ it flows out into the organ chamber (*O*) at approximately 0 mm. pressure. When the pressure of the equalization chamber (*E*) is approximately 0 mm., the fluid in the organ chamber (*O*) flows past a one-way floating valve (*F*) and into the equalization chamber (*E*). When the pressure in the equalization chamber (*E*) is 120 mm., the fluid flows past a second one-way floating valve (*F*) and into the reservoir chamber (*R*), thereby completing the cycle.

In actual operation, there is a back pressure in the organ chamber (*O*). Also, the lowest pressure in the gas line is always above 0 mm. This is due to the rapidity of pulsation, pressure of fluid columns, floating valves, etc.; and must be taken into consideration in adjusting the relative internal and external pressures on the organ.

### *Detailed Principles of Operation*

*Pulsation Rate.*—The pulsation rate is controlled by the speed of rotation of the pulsation valve (35). This valve is operated by an electric motor (34) which is geared down, and the speed of which can be controlled by a rheostat (33). There are two complete pulsation cycles per revolution of the valve.

*Pressure during Pulsation Cycle.*—The rate at which maximum pressure is reached and released, is controlled by the relative size of the slots in the pulsation valve rotor (62). Decreasing the length of the pressure slots (64) causes the maximum pressure to be reached more quickly. Control of pressure during the entire pulse cycle may be obtained by varying the length and width of the pressure and release slots in the rotor. Adjustments of this kind can be made only by stopping the apparatus and changing rotors. A relatively long release slot reduces the back pressure in the organ chamber, and a short release slot increases it.

*Sterility.*—Sterility in operation is maintained by passing the pulsating gas, which enters the perfusion pump, through two glass bulbs filled with non-absorbent cotton (12, 22). Two more glass bulbs filled with non-absorbent cotton (1, 2) protect the gas inlet and outlet to the organ chamber. No part of the apparatus beyond these filter bulbs is kept sterile. The filter bulbs and all parts of the apparatus on the organ side of them, are sterilized with heat.

After the perfusion fluid and the organ to be perfused have been aseptically inserted in the apparatus, all openings are closed with rubber stoppers, through which have been inserted glass tubes leading to the cotton filter bulbs. All joints between rubber and glass on the sterile side of the filter bulbs are sealed with waterproof cement. This leaves the only communication to the exterior protected by four cotton filter bulbs. All filter bulbs are placed in an approximately vertical position with the sterile opening high, to prevent the possibility of condensation water carrying infection through the filter.

*Temperature.*—A constant and controllable temperature is maintained by keeping the major portion of the apparatus in an incubator. The pulsating air enters the incubator immediately after leaving the pulsation valve (35) and passes

through a coil of copper tubing (38) to an oil flask (40). The copper coil (38) has a capacity greater than the volume of air moving back and forth through it during pulsation. Consequently, its narrow diameter prevents the rapid mixing of cool air from the pulsation valve (35) with the warm air from the oil flask (40). The control gas is brought up to incubator temperature as it passes through the tubing leading to the oil flask (40).

*Control of Gas in Contact with Perfusion Fluid.*—An oil flask (40) is used to separate the pulsating air, which actuates the apparatus, from the controlled gas which is in contact with the perfusion fluid and the organ. This flask (40) is made of one piece of pyrex glass with three openings to the outside (23, 24, 26). The pulsating air from the pulsation valve (35) passes through the lower opening (26) to the outer chamber (27). The pulsating control gas to the perfusion pump (49) passes through the middle opening (24) from the inner chamber (25). The fresh control gas from the steel gas cylinder (58) passes through the upper opening (23) to the inner chamber (25).

In operation, the pulsating pressure of the air from the pulsation valve (35) is transmitted through the oil to the control gas. Air is always in contact with the oil surface in the outer chamber (27), and control gas always in contact with the oil surface in the inner chamber (25). Fresh control gas passes through the opening (23) on top of the inner oil chamber (25). Its rate of flow is regulated by a Hoffman clamp (39) on the rubber gas line leading from the gas cylinder (58). The replaced gas bubbles out into the outer chamber (27) during the pressure release portion of the pulsation cycle and is carried away with the released air. In this way, the composition of the gas in contact with the perfusion fluid is kept constant. All surfaces of the fluid are in contact with controlled gas.

*Prevention of Evaporation.*—Evaporation of the perfusion fluid is prevented by maintaining the temperature of the oil flask (40), and the oil check valve and one-way valve assembly (41, 42, 43, 44, 45, 46), slightly higher than that of the perfusion pumps (49). In consequence, there is no movement of moisture away from the pumps, and whatever condensation there is takes place within the perfusion pumps. If this becomes appreciable, it runs down the walls and back into the perfusion fluid.

*Filming.*—Filming of the perfusion fluid is prevented by avoiding any bubbling of gas through the fluid, any spraying of the fluid, or any dropping of the fluid during its circulation. The perfusion pump is so constructed that the fluid always follows and adheres to a glass surface.

*Measurement of Rate of Flow.*—Rate of flow is measured by stopping the return circulation from the organ chamber (4), and opening the organ chamber to atmospheric pressure. Either the rate of rise of fluid in the organ chamber (4), or the rate of fall in the reservoir chamber (18) can be timed.

*Automatic Adjustment for Changes in Rate of Flow.*—The perfusion fluid in the cannula (3) leading to the organ is under a constant pulsating pressure due to the compression and release of the control gas. The pressure exerted by this control gas is practically unaffected by the rate of flow through the organ. Consequently,

as long as the capacity of the cannula, which is inserted in the artery of the organ, is materially greater than the rate of flow through the organ, the pressure exerted by the perfusion fluid is practically constant, regardless of changes in rate of flow through the organ.

*Filtration of the Perfusion Fluid.*—Two 150 mesh platinum screens (21) are located in the glass feed tube (20) which carries the perfusion fluid from the reservoir chamber to the organ. These screens are located at the top of the tube, and filter the fluid shortly before it enters the organ. After passing through the organ, the perfusion fluid is again filtered in passing through silica sand (6), held between two 52 mesh platinum screens (5).

*Separation of Organ and Perfusion Fluid from Non-Sterile Substance by One Piece of Glass and Sterile Control Gas.*—The perfusion pump is made of a single piece of glass except for the filters and floating glass valves (5, 6, 21, 9, 15). There are three openings to the outside. One of these is the mouth of the organ chamber (4); one is the neck (11) of the equalizing chamber (13); the third is the neck of the reservoir chamber (18). During operation the perfusion fluid does not come in contact with any of these openings. Consequently, the organ and perfusion fluid are separated by solid glass walls and sterile gas from all infected surfaces. Contact between the fluid and the outside is made only through the pulsating sterile control gas.

*Simplicity in Sterilization.*—The organ, equalization, and reservoir chambers (4, 13, 18) are separated from other parts of the apparatus by cotton filter bulbs (1, 2, 12, 22). No part of the apparatus beyond these filter bulbs is kept sterile.

*Aseptic Installation and Removal of Organs.*—The organ chamber (4) is placed at an angle approaching horizontal to reduce the mouth opening exposed to the air. The mouth is long enough to be flamed without injury to the organ and is sealed by a rubber stopper.

*Aseptic Installation and Removal of Perfusion Fluid.*—The necks to the reservoir and equalization chambers (18, 13) are of small diameter and can be flamed. A pipette can be inserted, through the neck of the reservoir chamber (18) to the bottom of the chamber, for removing the perfusion fluid.

### *Description of Construction and Operation*

*Perfusion Pump.*—The reservoir chamber (18) is under a pulsating pressure due to the compression and release of the control gas passing through the cotton filter bulb (22). Consequently, the perfusion fluid from the reservoir chamber (18) passes into the mouth of the feed tube (19), and through the feed tube (20). While passing through the feed tube (20) the perfusion fluid is filtered by two 150 mesh 0.0016 wire platinum screens (21). The fluid then passes through the cannula (3) to the organ. It exerts a pressure on the organ which is approximately equal to the pulsating pressure in the reservoir chamber (18), less the back pressure of the column in the feed tube (20) and the back pressure in the organ chamber (4). After passing through the organ, the fluid arrives in the organ chamber (4), which is under a pressure of approximately 20 mm. Hg (depending on pulsation rate,

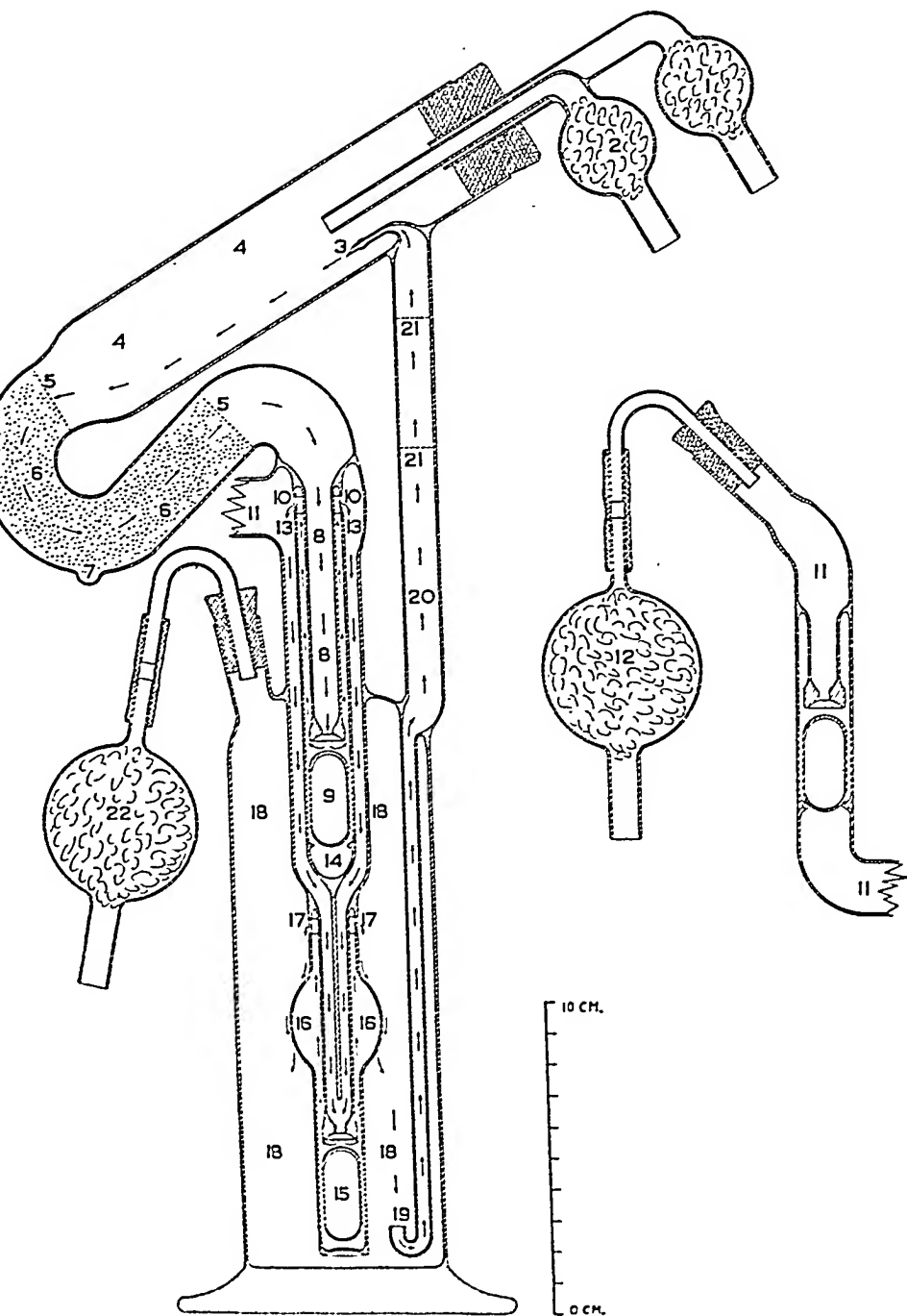


CHART 2. Cross-section of pulsating perfusion pump, (1-22).



pulsation pressures, diameter and lengths of tubing, depth of oil flask outer reservoir, volume of pulsating gas, etc.). When the pump is in operation, the organ chamber filter bulbs (1, 2) are closed. Consequently, the organ chamber is sealed gas tight.

During the portion of each pulsation in which the upper floating valve (9) is closed, the perfusion fluid collects in the organ chamber after passing through the organ. During the portion of each pulsation in which the upper floating valve (9) is open, the fluid from the organ chamber passes (partly due to gravity and partly to the added pressure its volume has created in the organ chamber) through the 52 mesh 0.004 wire platinum screen (5), through the silica sand filter (6), through the second platinum 52 mesh screen (5), down through the inner tube (8), past the upper floating valve (9) seat, up the floating valve reservoir (14), through the holes (10) at the top of the floating valve reservoir (14), and into the equalization chamber (13).

The perfusion fluid remains in the equalization chamber (13) during the portion of each pulsation cycle in which the lower floating valve (15) is closed. During the portion of each pulsation cycle in which the lower floating valve (15) is open, the fluid in the equalization chamber flows (partly due to gravity and partly to the relative rate of pressure equalization in the reservoir and equalization chambers) down past the lower floating valve (15) seat, up through the floating valve reservoir (16), through the holes (17) at the top of the floating valve reservoir (16), and into the main reservoir chamber (18), thereby completing the cycle of circulation.

The opening and closing of the floating valves (9, 15) is regulated by the pressure in the equalization chamber (13), relative to the pressure in the reservoir chamber (18) and the organ chamber (4). When the pressure in the equalization chamber is minimum, and approximately equal to the pressure in the organ chamber, the upper floating valve (9) is open and the lower floating valve (15) is closed. When the pressure in the equalization chamber is maximum, and approximately equal to the pressure in the reservoir chamber, the lower floating valve (15) is open and the upper floating valve (9) is closed.

The pulsating gas passes back and forth through the filter bulbs (22, 12) leading to the reservoir and equalization chambers (18, 13). It exerts its maximum pressure on both chambers (18, 13) almost equally. Its minimum pressure is exerted only on the equalization chamber (13) because the outflow from the reservoir chamber (18) is restricted by a one-way gas valve (44) and adjustable constricted bypass (45). Consequently, under normal operation, the pressure in the reservoir chamber (18) never drops as low as the pressure in the equalization chamber (13).

The rate of flow of pulsating gas, in and out of the equalization chamber (13), is adjusted by means of a Hoffman clamp (48) placed on the rubber tube gas line leading to the equalization chamber filter bulb (12). This adjustment is necessary because of the small gas capacity of the equalization chamber (13) in relation to the gas capacity of the reservoir chamber (18). Without adjustment, the pulsating gas fills the equalization chamber more quickly, and raises its pressure

enough above that in the reservoir chamber to push gas past the floating valve (15), separating the two chambers (13, 18), and cause bubbling and filming in the reservoir chamber (18).

The floating valve in the equalization chamber neck (11) is for the purpose of stopping an overflow of the perfusion fluid if the lower floating valve (15), separating the equalization (13) and reservoir (18) chambers, becomes jammed due to the consistency of the perfusion fluid, or to infection, or to a fragment of degenerated tissue. When the fluid level in the equalization chamber (13) reaches and closes the floating valve in the equalization chamber neck (11), the entire pressure difference between the minimum pressure in the reservoir chamber (18) and the maximum pressure in the equalization chamber (13), plus the weight of the fluid column in the equalization chamber and neck (13, 11), is exerted on the jammed lower valve (15).

The filter bulbs (1, 2), leading to the organ chamber, are to permit the sterile gassing of the chamber with control gas. The organ chamber is of tubular construction for simplicity, and to permit sealing the mouth with a rubber stopper. It is set at an angle of about 30 degrees with the horizontal to maintain a slight tension on the artery leading to the organ, to prevent the perfusion fluid from touching the rubber stopper, and to simplify flaming and the aseptic installation of the organ. The large size of all external glass tubing is for strength of construction.

The open end of the equalization chamber neck (11) is set at an angle which permits the introduction of perfusion fluid and still minimizes the possibility of infection. The neck of the reservoir chamber (18) is set at an angle which permits the insertion of a pipette to the bottom of the chamber for the purpose of withdrawing the perfusion fluid. The filter bulbs leading to the equalization and reservoir chambers are flexibly connected by rubber tubing to lessen the possibility of breakage in handling. The floating valves are ground with a round, rather than a flat, seat. The guide tubes, in which they operate, and which form a reservoir for the perfusion fluid in which they float, have an inside diameter of 1 mm. to 1.2 mm. greater than the outside diameter of the floating valve. Too little clearance imprisons gas below the valve and makes the action sluggish. Too great clearance or a flat seat prevents proper closing.

The aperture in the valve seat is about 3 mm. The ground portion of the seat is of sufficient area to prevent leakage. The holes at the top of the floating valve reservoir (10, 17) are large (about 5 mm. diameter) to prevent spurting of the perfusion fluid. There are four holes at the top of each reservoir.

The clearance, between the outside of the valve reservoir tube (14) and the inside of the equalization chamber (13), is sufficient to prevent the fluid, which passes through the holes (10) at the top of the valve reservoir (14), from touching the inner wall of the equalization chamber (13). The perfusion fluid should run down the outer surface of the valve reservoir tube (14). If it touches the inner surface of the equalization chamber (13), filming may occur. A solid glass rod at the bottom of the floating valve reservoir (14) guides the fluid to the lower

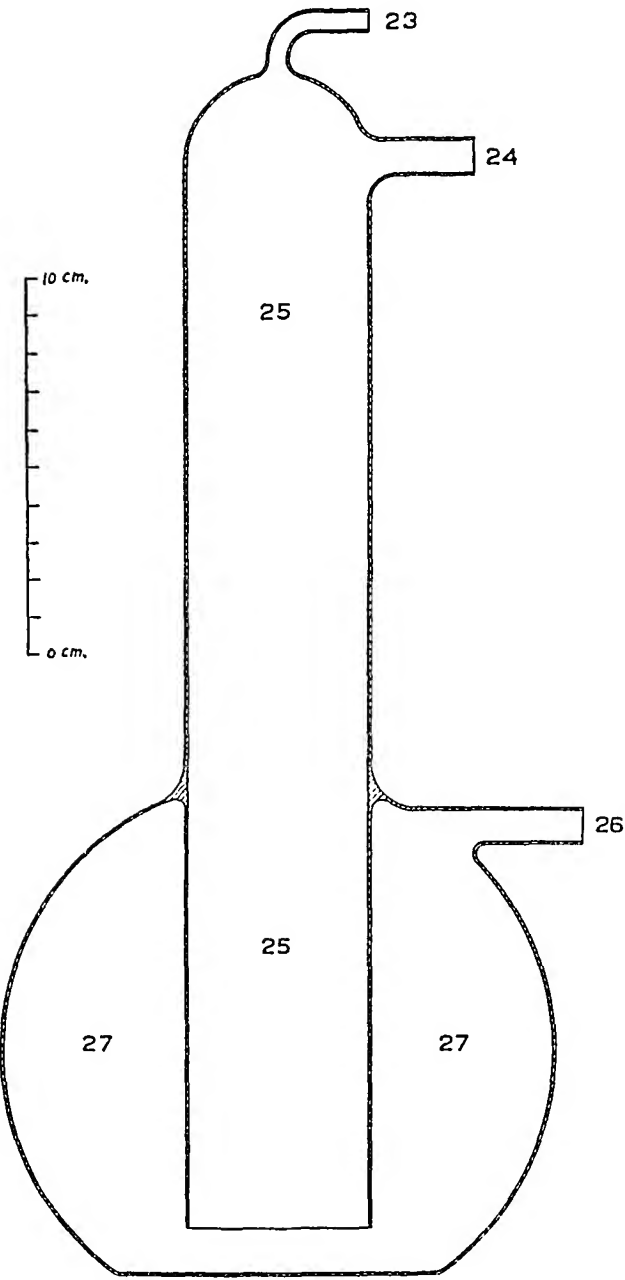


CHART 3. Cross-section of oil flask, (23-27).

portion of the equalization chamber, and prevents filming if the fluid level in the equalization chamber (13) is too low due to improper adjustment.

The lower floating valve reservoir tube (16) is blown out to a wider diameter near the middle. This is to prevent the rapid emptying of the tube if the valve does not always seat perfectly, and to keep all the fluid from being blown out of the floating valve reservoir while the rate of flow of pulsating gas to the equalization chamber is being adjusted. The lower position of the lower floating valve (15) is governed by making the end of the valve reservoir tube (16) concave. The lower position of the other two floating valves is governed by three indentations made around the glass guide tubing. From 3 to 4 mm. is sufficient range of valve travel. The inlet (19) of the feed tube (20) is bent upward, to prevent the loss of perfusion fluid from the feed tube (20) while the fluid in the reservoir chamber is being changed.

Crushed quartz sand is used in the filter (6). The sand must be sufficiently coarse to prevent its passing through the 52 mesh platinum screens (5). All dust and small particles of sand are thoroughly washed out before it is placed in the pump. Then the glass tube (7), through which the sand is inserted, is sealed.

*Oil Flask.*—The oil flask (40) is made of pyrex glass and contains two chambers (25, 27). The outer chamber (27) is partially filled with oil (liquid petrolatum U.S.P.). Air passing the pulsation valve (35) enters the outer chamber (27), and creates a pressure in the outer chamber which forces the oil into the inner chamber (25), thereby compressing the control gas in the inner chamber and transmitting pressure from the air pressure line to the perfusion pumps (49). When the pulsation valve (35) is in the release position, the compressed control gas in the inner chamber (25) forces the oil back into the outer chamber (27), thereby completing the pulsation cycle. Fresh control gas enters the top opening of the inner chamber (23). The replaced control gas bubbles from the inner chamber into the outer chamber during the release portion of the pulsation cycle, and is carried away through the release outlet (65) of the pulsation valve (35). The oil flask should be filled to a level which will not cause overflow from the outer chamber (27) when gas is bubbling from the inner chamber (25) into the outer chamber.

*Pulsation Valve.*—The pulsation valve (35) consists of a steel rotor (62) turning inside of a stationary bronze cylinder (60). During one portion of rotation the valve permits air from the air pressure line to pass into the oil flask outer chamber (27). During another portion of rotation, it releases the air from the outer chamber (27) of the oil flask. The valve is so constructed that each rotation causes two complete pulsation cycles. The pressure during the pulsation cycle may be governed by changing the lengths and widths of the slots (63, 64), milled in the steel rotor (62). For example, shortening the pressure slots (64) causes the maximum pressure to be reached more quickly and reduces the average pressure of the cycle.

*Oil Check Valve.*—The oil check valve (41) contains a sealed glass cylinder (59) with a slender taper at one end. This cylinder (59) is held in a second glass cylinder (41), of a diameter sufficient to permit the free flow of pulsating gas

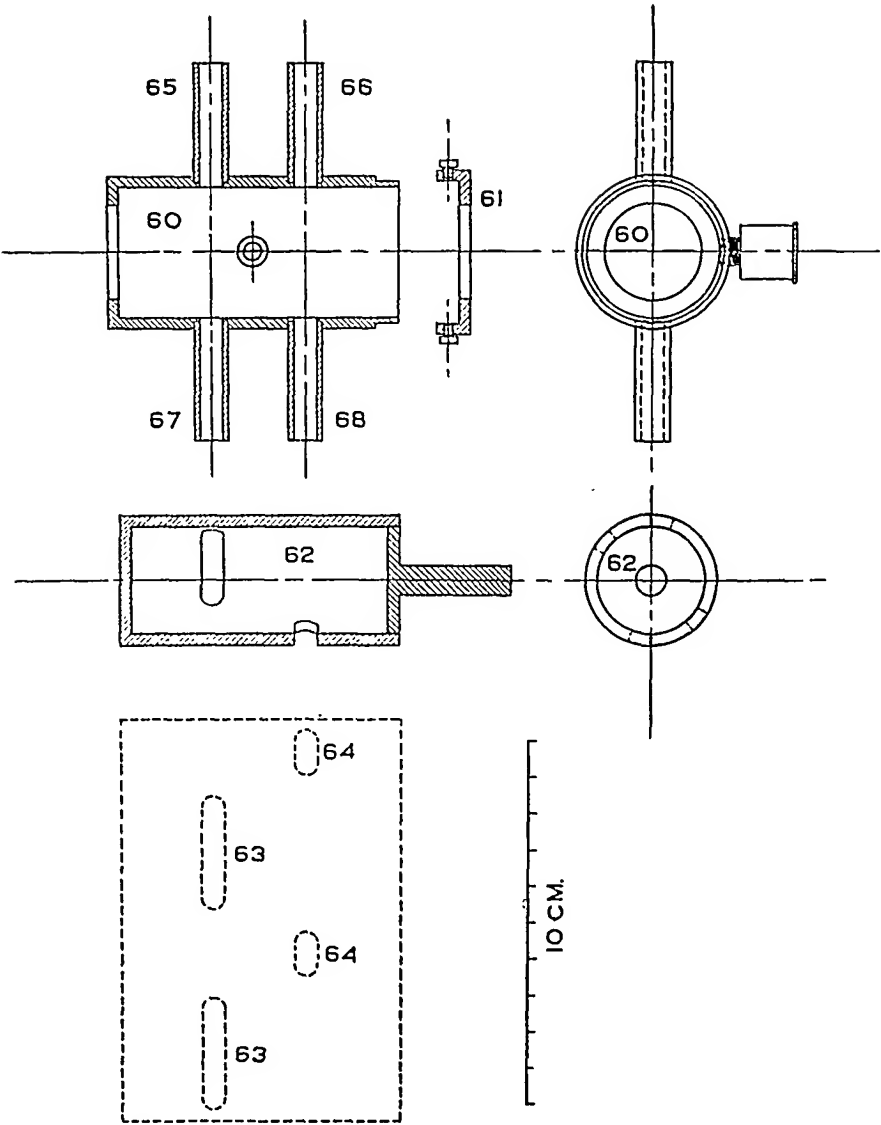


CHART 4. Pulsation valve, (60-68).

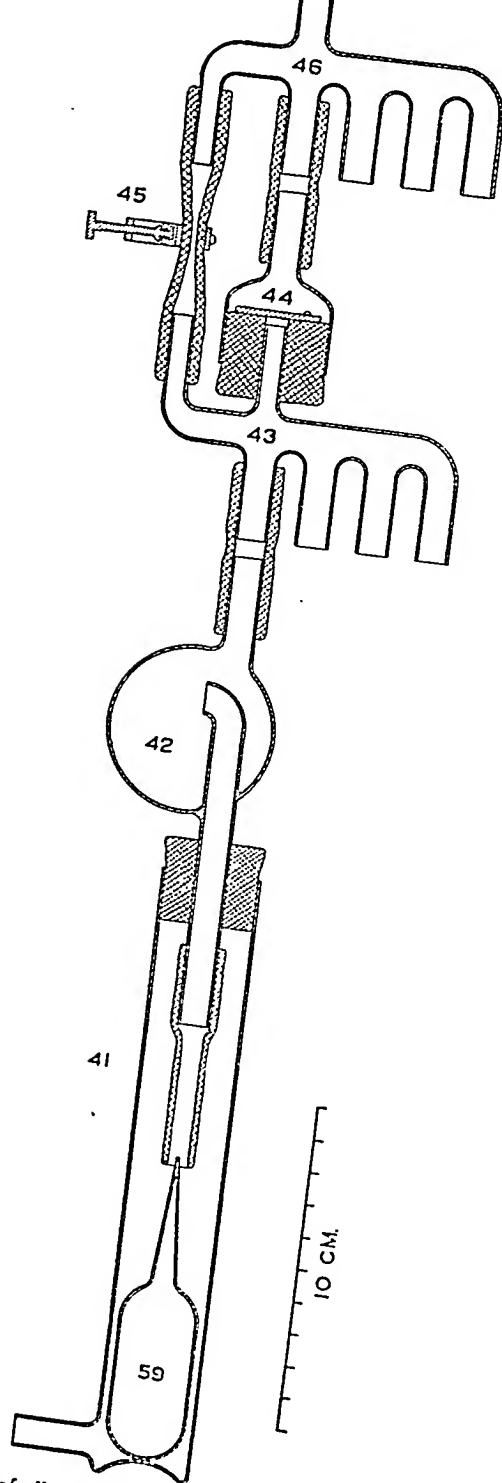


CHART 5. Cross-section of oil check valve, oil trap, and one-way gas valve assembly, (41-46, 59).

between the walls of the two cylinders (41, 59). The top of the outer cylinder (41) is closed with a rubber stopper, through the center of which runs a glass tube. A short length of rubber tubing is connected to the inside end of this glass tube. It is so adjusted that, when the rubber stopper is in place, the tapered end of the sealed inner cylinder (59) enters the end of the rubber tubing about 5 mm. The tapered end of the sealed inner cylinder (59) is covered with vaseline before assembling. The lower end of the outer cylinder (41) is connected by rubber tubing to the middle opening of the oil flask (24). The upper end of the outer cylinder (41) is connected directly to the oil trap (42).

The pulsating gas passes freely back and forth through the oil check valve (41); but if oil overflows the oil flask inner chamber (25), it flows into the bottom opening of the oil check valve (41), and floats the inner cylinder (59) until the tapered end closes the rubber tubing in which it is inserted, thereby preventing the oil from passing farther into the system.

*Oil Trap.*—The oil trap (42) is made of glass and is directly above the oil check valve (41). It is for the purpose of preventing any leakage of oil, past the oil check valve (41), from passing farther into the system.

*One-Way Gas Valve.*—The one-way gas valve (44) is made of glass, rubber, and metal pins. A hole (about 6 mm. diameter) is drilled through the center of a rubber stopper. A rubber flap is placed over this hole on the inside of the stopper and pinned at one edge. The rubber stopper is then inserted in the large end of the glass tube (44). This forms a one-way valve which permits the pulsating gas to pass in one direction only.

### *Assembly of Apparatus*

The incubator used should have the heating element under the floor. It should have an inner glass door to permit observation of the interior without cooling the apparatus.

The perfusion pumps (49) should be placed in the front part of the incubator with the organ chamber mouths pointing toward the rear. They should be placed on a platform which thoroughly insulates them from the hot floor of the incubator. A sheet of asbestos on legs, which raise it about 3 cm. above the incubator floor, is satisfactory. This stand should be narrow enough so that the oil flask (40) can be placed directly on the hot floor of the incubator near the rear. The oil check valve and one-way valve assembly (41, 42, 43, 44, 45, 46) should be hung in a vertical position on the side wall of the incubator and near the rear. It should be placed to minimize the length of rubber tubing required to connect the oil check valve (41) to the oil flask (40). It is desirable to minimize the length of tubing used wherever possible throughout the apparatus.

The coil of copper tubing (38) should be placed directly on the hot floor of the incubator, next to the side or rear wall, and immediately adjoining the oil flask (40). This coil (38) should have a minimum inside diameter of 10 cm. The tubing should have a minimum inside diameter of 8 mm. The capacity of the coil must be greater than the volume of gas which pulsates back and forth through

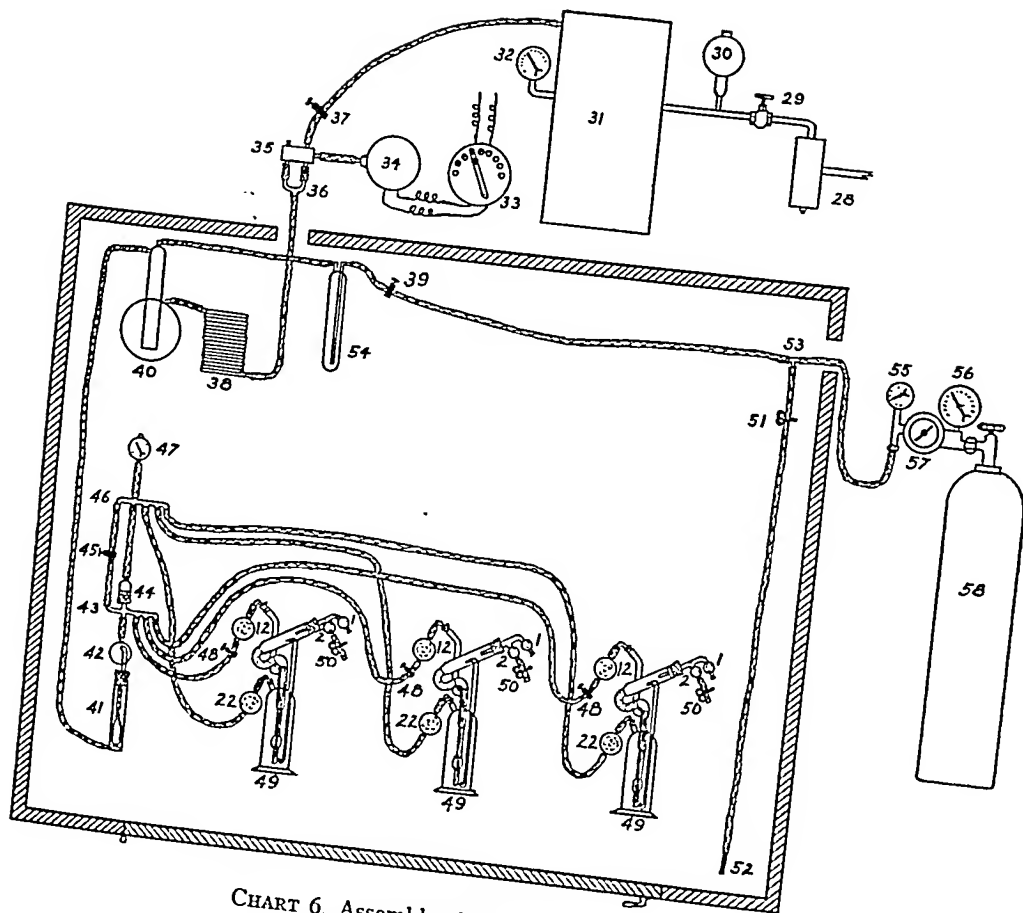


CHART 6. Assembly sketch, (1, 2, 12, 22, 28-58).



it. It is essential that the perfusion pumps (49) be placed in the coolest part of the incubator, so that the temperature of the oil flask (40) and the oil check valve and one-way valve assembly (41, 42, 43, 44, 45, 46) be higher than that of the pumps (49).

The pressure gauge (47) may be placed near the front of the incubator and connected with a longer tube, if desired. The bottom of the copper coil (38) is connected by rubber tubing to a glass Y (36), which is connected by two rubber tubes to the pressure outlet (68) and relief inlet (67) of the pulsation valve (35). The pulsation valve (35) should be placed close to the outside wall of the incubator, opposite the copper coil (38). A hole is drilled through the wall for the connecting tubing.

The pulsation valve (35) is connected by a flexible shaft to a 1/24 h.p. electric motor (34), which is geared down. The motor (34) is controlled by a rheostat (33) and so geared that the desired range of pulsation rate can be obtained. There are two complete pulsation cycles with each revolution of the pulsation valve (35).

The relief outlet (65) of the pulsation valve (35) can be left open or carried into a muffler, if desired. A satisfactory muffler can be made by coating the inside of a glass jar with the same cement used for sealing the corks on the perfusion pumps, and then lining the jar with non-absorbent cotton. If a muffler is used, care must be taken not to create excessive back pressure on the relief outlet (65) of the pulsation valve (35).

The pressure inlet (66) of the pulsation valve (35) is connected to an air reservoir tank (31). This tank should have sufficient capacity to avoid more than 1 cm. Hg change in pressure during each pulsation. A pressure gauge (32) is connected to the air reservoir tank (31). A pressure relief valve (30) is connected to the air line leading to the air reservoir tank (31). This relief valve should have sufficient capacity to minimize any changes of pressure in the air pressure line or changes of flow through the pulsation valve. A needle valve (29) is located on the air pressure line beyond this relief valve (30). Beyond the needle valve (29) is located a dirt and water trap (28) which contains a copper screen in the top, and a drain plug in the bottom. This trap is connected to the outside air pressure line.

The oil check valve and one-way valve assembly (41, 42, 43, 44, 45, 46) must be placed in a vertical position. When three perfusion pumps (49) are run on one system, a six-way glass fitting (43) is placed above the oil trap (42). One tube connects this fitting (43) to the oil trap (42); a second tube connects it to the one-way valve (44); a third, to the constricted opening (45) which bypasses the one-way valve (44). The remaining three tubes lead to the equalization chamber filter bulbs (12) of the three perfusion pumps (49).

A second six-way glass fitting (46) is placed above the one-way valve (44). One tube connects this fitting (46) to the one-way valve (44); a second tube connects it to the constricted opening (45); a third, to the pressure gauge (47). The remaining three tubes lead to the reservoir chamber filter bulbs (22) of the three perfusion pumps (49).

The one-way gas valve (44) is placed between the two six-way glass fittings (43, 46), and must be in a vertical position. The direction of flow is upward. The constricted opening (45) is formed and adjusted by a Hoffman clamp placed on a rubber tube which bypasses the one-way valve (44). Hoffman clamps (48) are placed on each of the rubber tubes leading from the lower six-way fitting (43) to the equalization chamber filter bulbs (12) of the perfusion pumps (49). These clamps (48) should be placed at a high point in the line, either near the filter bulb (12) or near the six-way fitting (43). This is to avoid possible interference by condensation water.

Controlled gas is led into the incubator from a steel gas cylinder (58). A gas pressure regulator valve (57) is used to reduce the pressure at the cylinder outlet. The two gauges (55, 56) attached to this gas regulator, indicate the pressure in the cylinder and the pressure at the gas outlet. A rubber tube leads from the gas outlet into the incubator to a glass T (53). From this T (53) one tube leads to a saturation flask (54) where the gas is bubbled through water. From the saturation flask (54) a rubber tube leads to the upper opening of the oil flask inner chamber (23). A Hoffman clamp (39) is placed next to the saturation flask (54) on the gas inlet rubber tube. A second rubber tube from the glass T (53) leads to a capillary glass tube (52) (about  $\frac{1}{2}$  mm. opening and 10 cm. long). This rubber tube is long enough to permit the capillary glass tube (52) to be inserted in the short rubber tube which is connected to the inner filter bulb (2) of the organ chamber (4) of the most distant perfusion pump (49). When not in use, it is closed by a Day pinch-cock (51).

The air reservoir tank (31) is connected to the outside air pressure line by  $\frac{1}{2}$  inch metal pipes and fittings. The rubber tubing used between the air reservoir tank (31) and the oil check valve (41) has a minimum inside diameter of 7 mm. and an outside diameter of 14 mm. The rubber tube inside the oil check valve (41) has an inside diameter of 6 mm. and an outside diameter of 10 mm. The rubber tubes, connecting the oil trap (42) to the lower six-way glass fitting (43), and connecting the one-way gas valve (44) to the upper six-way glass fitting (46), have an inside diameter of 7 mm. and an outside diameter of 14 mm. The rubber tube which bypasses the one-way gas valve (44), and which is used to form the constricted opening (45), has an inside diameter of 4 mm. and an outside diameter of 13 mm. All rubber tubes leading from the two six-way glass fittings (43, 46) to the perfusion pumps (49) have an inside diameter of 6 mm. and an outside diameter of 10 mm. Similar tubing is used on the inner filter bulbs (2) of the perfusion pumps (49), and to connect the upper six-way fitting (46) to the pressure gauge (47). The rubber tubing, between the control gas cylinder (58) and the oil flask (40) and capillary glass tube (52), has an inside diameter of 3 mm. and an outside diameter of 7 mm.

It is desirable to have all rubber tubing elastic enough to simplify making the connections between rubber and glass. However, it should not be so elastic that its diameter changes appreciably with the pulsations. Too small a diameter of the tubing, carrying the pulsating gas, will cause high back pressure and ineffi-

cient operation of the apparatus. Wherever practicable the glass tubing used in the pulsation line has an inside diameter of at least 8 mm.

### *Operating Directions<sup>3</sup>*

*Washing Perfusion Pump.*—Rinse the pump with water. Place a rubber stopper, with a glass tube through its center, in the mouth of the organ chamber. Attach a rubber hose to the glass tube, and force water, under low pressure, through the sand filter (6) and cannula (3). Pour the excess water out of the pump and fill with chromic and sulfuric acid. The reservoir chamber neck must be closed with a rubber stopper when it is full, and then the upper portions of the pump filled with acid. The acid can be distributed through the sand filter more quickly by filling the organ chamber and closing it with a cork, which has a glass tube through the center. A light air pressure on this glass tube will force the acid quickly through the sand. After the acid has been in the pump for about 2 hours, pour it out and rinse the pump with water. All washing water, run through the pump after the acid has been removed, must be thoroughly filtered. After rinsing, place the rubber stopper, containing a glass tube through its center, in the mouth of the organ chamber; and pass water under low pressure through the pump for about 1 hour. It is essential that sufficient time be given for all organic material to be removed by the acid, and for all acid to be removed by the washing water. Pour out the water and dry the pump by vacuum. The equalization chamber neck should be partially closed by a cork with a small hole through its center. The vacuum hose is connected to the neck of the reservoir chamber. Drying should continue until the moisture is removed from all parts, including the sand filter. Care must be taken that the vacuum used is not strong enough to cause the floating valves to chatter. They chatter more easily when dry than when wet. Consequently, a negative pressure which does not cause chatter at first, may cause destructive chatter as the valves become dry. After the pump is dry, it is sterilized in dry heat. The glass filter bulbs, which are lightly packed with non-absorbent cotton, and which are attached to rubber, are sterilized in steam heat.

*Preparing for Organ Installation and Sealing Pump after Installation.*—After installation of the perfusion fluid, and prior to installation of the organ, a rubber tube is attached to the reservoir chamber filter bulb (22); and enough air pressure placed in the reservoir chamber (18) to force perfusion fluid through the cannula (3). Sufficient fluid is blown into the organ chamber (4) to wet the sand filter (6) and fill the upper floating valve reservoir (14). Then while perfusion fluid is still passing through the cannula (3), a pinch-cock is placed on the rubber tube connected to the reservoir chamber filter bulb (22). This holds the perfusion fluid level even with the mouth of the cannula (3), while the organ is being in-

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<sup>3</sup> Great assistance has been received from J. Zwick in developing the technique connected with the operation of this apparatus.

stalled. After the organ has been installed in the pump, a rubber stopper, containing tubes leading to the filter bulbs (1, 2), is placed in the organ chamber mouth. All stoppers and joints between glass and rubber on the sterile side of the filter bulbs are sealed with waterproof cement (DuPont clear dope No. 5332).

*Starting Apparatus Preparatory to Installing Perfusion Pump.*—Be sure that pinch-cocks are on all pump gas line tubes. Turn on the gas cylinder (58) valve and adjust the gas regulator to about 8 pounds outlet pressure. Open the clamp (39) which adjusts the flow of control gas into the oil flask inner chamber (25), until gas starts to bubble out into the outer chamber of the oil flask (27). Then close the clamp (39). If the oil level in the inner chamber of the oil flask (25) is too high when pulsation is started, the oil will overflow and close the oil check valve. Make sure that the clamp (37), on the air line leading to the pulsation valve (35), is closed. Set the air release valve (30) to release at about 8 pounds pressure, as indicated on the air reservoir tank gauge (32). Adjust the air line needle valve (29) until there is an appreciable leak through the release valve (30). Switch on the pulsation valve motor (34) and adjust the rheostat (33) to the desired pulsation rate. Open the clamp (37), on the air line leading to the pulsation valve (35), until the pressure gauge (47) indicates the maximum pressure desired in the reservoir chamber (18). Adjust the clamp (39), controlling the flow of control gas to the oil flask inner chamber (25), until the desired flow is obtained as indicated by the rate of bubbling through the saturation flask (54). Shut the clamp on the pressure gauge (47) gas line (to save wear on the gauge and to permit its removal and use on other systems).

*To Install Perfusion Pump in Incubator.*—Place the short rubber tube on the inner organ chamber filter bulb (2). Seal with waterproof cement. Place the pump in the incubator with the organ chamber mouth to the rear. Close the clamp (39) on the rubber gas line leading from the steel gas cylinder (58) to the oil flask inner chamber (25). Connect the capillary glass tube (52) to the rubber tube on the organ chamber inner filter bulb (2); and allow gas to pass through the organ chamber by removing the pinch-cock (51) from the capillary tube (52) gas line. After 2 or 3 minutes replace the pinch-cock (51) on the capillary tube gas line and disconnect the capillary glass tube from the organ chamber filter bulb rubber tube. Close the organ chamber inner filter bulb (2) rubber tube with a pinch-cock (50). Close the organ chamber outer filter bulb (1) with a rubber stopper. Seal with waterproof cement. Connect the gas tube from the six-way glass fitting (46), above the one-way valve (44), to the reservoir chamber filter bulb (22). Connect the gas tube from the six-way glass fitting (43), below the one-way valve (44), to the equalization chamber filter bulb (12). Connection of tubing to filter bulbs is simplified if the glass is moistened with water. Remove the pinch-cock from the gas line leading to the reservoir chamber (18). Remove the pinch-cock from the gas line leading to the equalization chamber (13). Make sure there is a slight leak past the air line relief valve (30). Set the maximum pressure by adjusting the clamp (37) on the air line leading to the pulsation valve (35). Set the minimum pressure by adjusting the clamp (45) which controls the constricted

cient operation of the apparatus. Wherever practicable the glass tubing used in the pulsation line has an inside diameter of at least 8 mm.

### *Operating Directions*<sup>3</sup>

*Washing Perfusion Pump.*—Rinse the pump with water. Place a rubber stopper, with a glass tube through its center, in the mouth of the organ chamber. Attach a rubber hose to the glass tube, and force water, under low pressure, through the sand filter (6) and cannula (3). Pour the excess water out of the pump and fill with chromic and sulfuric acid. The reservoir chamber neck must be closed with a rubber stopper when it is full, and then the upper portions of the pump filled with acid. The acid can be distributed through the sand filter more quickly by filling the organ chamber and closing it with a cork, which has a glass tube through the center. A light air pressure on this glass tube will force the acid quickly through the sand. After the acid has been in the pump for about 2 hours, pour it out and rinse the pump with water. All washing water, run through the pump after the acid has been removed, must be thoroughly filtered. After rinsing, place the rubber stopper, containing a glass tube through its center, in the mouth of the organ chamber; and pass water under low pressure through the pump for about 1 hour. It is essential that sufficient time be given for all organic material to be removed by the acid, and for all acid to be removed by the washing water. Pour out the water and dry the pump by vacuum. The equalization chamber neck should be partially closed by a cork with a small hole through its center. The vacuum hose is connected to the neck of the reservoir chamber. Drying should continue until the moisture is removed from all parts, including the sand filter. Care must be taken that the vacuum used is not strong enough to cause the floating valves to chatter. They chatter more easily when dry than when wet. Consequently, a negative pressure which does not cause chatter at first, may cause destructive chatter as the valves become dry. After the pump is dry, it is sterilized in dry heat. The glass filter bulbs, which are lightly packed with non-absorbent cotton, and which are attached to rubber, are sterilized in steam heat.

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stalled. After the organ has been installed in the pump, a rubber stopper, containing tubes leading to the filter bulbs (1, 2), is placed in the organ chamber mouth. All stoppers and joints between glass and rubber on the sterile side of the filter bulbs are sealed with waterproof cement (DuPont clear dope No. 5332).

*Starting Apparatus Preparatory to Installing Perfusion Pump.*—Be sure that pinch-cocks are on all pump gas line tubes. Turn on the gas cylinder (58) valve and adjust the gas regulator to about 8 pounds outlet pressure. Open the clamp (39) which adjusts the flow of control gas into the oil flask inner chamber (25), until gas starts to bubble out into the outer chamber of the oil flask (27). Then close the clamp (39). If the oil level in the inner chamber of the oil flask (25) is too high when pulsation is started, the oil will overflow and close the oil check valve. Make sure that the clamp (37), on the air line leading to the pulsation valve (35), is closed. Set the air release valve (30) to release at about 8 pounds pressure, as indicated on the air reservoir tank gauge (32). Adjust the air line needle valve (29) until there is an appreciable leak through the release valve (30). Switch on the pulsation valve motor (34) and adjust the rheostat (33) to the desired pulsation rate. Open the clamp (37), on the air line leading to the pulsation valve (35), until the pressure gauge (47) indicates the maximum pressure desired in the reservoir chamber (18). Adjust the clamp (39), controlling the flow of control gas to the oil flask inner chamber (25), until the desired flow is obtained as indicated by the rate of bubbling through the saturation flask (54). Shut the clamp on the pressure gauge (47) gas line (to save wear on the gauge and to permit its removal and use on other systems).

*To Install Perfusion Pump in Incubator.*—Place the short rubber tube on the inner organ chamber filter bulb (2). Seal with waterproof cement. Place the pump in the incubator with the organ chamber mouth to the rear. Close the clamp (39) on the rubber gas line leading from the steel gas cylinder (58) to the oil flask inner chamber (25). Connect the capillary glass tube (52) to the rubber tube on the organ chamber inner filter bulb (2); and allow gas to pass through the organ chamber by removing the pinch-cock (51) from the capillary tube (52) gas line. After 2 or 3 minutes replace the pinch-cock (51) on the capillary tube gas line and disconnect the capillary glass tube from the organ chamber filter bulb rubber tube. Close the organ chamber inner filter bulb (2) rubber tube with a pinch-cock (50). Close the organ chamber outer filter bulb (1) with a rubber stopper. Seal with waterproof cement. Connect the gas tube from the six-way glass fitting (46), above the one-way valve (44), to the reservoir chamber filter bulb (22). Connect the gas tube from the six-way glass fitting (43), below the one-way valve (44), to the equalization chamber filter bulb (12). Connection of tubing to filter bulbs is simplified if the glass is moistened with water. Remove the pinch-cock from the gas line leading to the reservoir chamber (18). Remove the pinch-cock from the gas line leading to the equalization chamber (13). Make sure there is a slight leak past the air line relief valve (30). Set the maximum pressure by adjusting the clamp (37) on the air line leading to the pulsation valve (35). Set the minimum pressure by adjusting the clamp (45) which controls the constricted

opening bypassing the one-way valve (44). Set the fluid level in the equalization chamber (13) by adjusting the clamp (48) on the gas line leading to the equalization chamber filter bulb (12). This level should be from 5 to 8 cm. above the holes (17) at the top of the lower floating valve reservoir (16). If the fluid level in the organ chamber is too high, insert the capillary glass tube (52) in the organ chamber inner filter bulb rubber tube. Remove the pinch-cock (50) from the inner filter bulb rubber tube, and allow gas to pass into the organ chamber by quickly opening and closing the pinch-cock (51) on the capillary tube (52) gas line, until the fluid level is satisfactory. Then replace the filter bulb pinch-cock (50) and disconnect. Readjust the clamp (39) on the rubber gas line leading from the gas cylinder (58) to the oil flask inner chamber (25).

The clamp (39) on the control gas line, leading from the gas cylinder (58) to the oil flask inner chamber (25), must be closed before gassing the organ chamber. Otherwise the fall in pressure in the control gas line during gassing would permit the escape of gas from the oil flask inner chamber (25), and consequent overflow of oil and closing of the oil check valve (41).

The pinch-cock on the reservoir chamber (18) gas line is removed before that on the equalization chamber (13) gas line, to avoid the violent bubbling which occurs if the reverse procedure is followed.

A slight leak past the air line release valve (30) is desirable to compensate for possible variations of pressure in the air line.

When minimum pressure is adjusted it is usually necessary to readjust the maximum pressure. A satisfactory procedure is, first, to set the maximum pressure; then to set the minimum pressure to the desired pressure difference between maximum and minimum, rather than to the actual minimum desired; then to make a final adjustment of maximum pressure. The pressure difference will remain approximately the same with minor adjustments of maximum pressure.

For a time after a pump has been installed, it is desirable to increase the flow of control gas into the oil flask inner chamber (25), in order to replace the air in the pump. After sufficient time has elapsed for the gas composition to become constant, the rate of bubbling may be decreased.

*To Remove Perfusion Pump from Incubator.*—Place a pinch-cock on the rubber gas line leading to the equalization chamber filter bulb (12). Place a pinch-cock on the rubber gas line leading to the reservoir chamber filter bulb (22). Disconnect the rubber gas line from the reservoir chamber filter bulb (22). If other pulsation pumps are on the system, readjust the pulsation pressure and the fluid level in their equalization chambers. Disconnect the rubber gas line from the equalization chamber filter bulb (12). Remove the pump from the incubator.

A pinch-cock should always be placed on the equalization chamber rubber gas line before one is placed on the reservoir chamber line. The reservoir chamber should be disconnected immediately after the two pinch-cocks are in place, and before the equalization chamber gas line is disconnected. The reverse procedure will cause bubbling.

*Rate of Flow Measurement.*—Rate of flow through the cannula leading to the

organ is measured with the organ chamber open to atmospheric pressure. Therefore, there is no back pressure in the organ chamber, and the effective pressure within the organ rises an amount equal to the previous back pressure in the organ chamber. Consequently, to measure the rate of flow at normal operating pressures, it is necessary to reduce the pressure in the reservoir chamber an amount equal to the back pressure in the organ chamber.

Measure the back pressure in the organ chamber (4) by connecting the pressure gauge to the rubber tube, which is on the organ chamber inner filter bulb (2). Reduce the pressure on the reservoir chamber (18) an amount equal to the back pressure in the organ chamber, by adjusting the clamp (37) on the air line leading to the pulsation valve (35). Place a pinch-cock on the gas line leading to the equalization chamber filter bulb (12). Open the organ chamber to atmospheric pressure by removing the pinch-cock (50) from the rubber tube on the inner filter bulb (2). Readjust the pressure in the reservoir chamber (18). Time either the rate of rise of fluid in the organ chamber or the rate of fall of fluid in the reservoir chamber. If the rate of flow was measured by the fluid rise in the organ chamber, check the fluid level in the upper floating valve reservoir (14). If the rate of flow was measured by the rate of fall in the reservoir chamber, check the fluid level in the lower floating valve reservoir (16). If the fluid level has fallen in the floating valve reservoir, due to leakage past the valve seat, it is necessary to make a corresponding correction in computing the rate of flow through the organ cannula. Replace the pinch-cock (50) on the inner filter bulb (2) rubber tube. Remove the pinch-cock from the rubber tube leading to the equalization chamber filter bulb (12). Readjust the pressure on the reservoir chamber (18) to the original amount. Adjust the level of the fluid in the organ chamber (4) by letting in control gas.

*Daily Inspection during Operation.*—Check the pulsation pressures on the pressure gauge (47). Check the fluid level in the organ chamber (4). Check the fluid level in the equalization chamber (13). Check the flow of control gas to the oil flask inner chamber (25). Check the oil level in the oil flask inner chamber (25), to be sure the flow of control gas is sufficient to maintain a slow bubbling of gas from the inner to the outer chamber (27) of the oil flask. One bubble in several pulsations is sufficient. Check the amount of gas in gas cylinder (58). Check the outlet pressure on the gas regulator valve outlet gauge (55). Check the air line relief valve (30) pressure. Make sure there is an appreciable air leak past the relief valve (30). Check the reservoir and equalization chamber filter bulbs (12, 22) for condensation water. Condensation water may accumulate in these bulbs if the incubator door is opened frequently. If necessary, flame the bulbs sufficiently to remove the water. Grease the pulsation valve (35) by turning down the pulsation valve grease cup. Keep the pulsation valve motor (34) properly oiled and the reduction gear box packed with grease.

*Stopping Apparatus after Removal of Perfusion Pumps.*—Shut the clamp (39) on the control gas line leading to the oil flask inner chamber (25). Gradually close the clamp (37) on the air pressure line leading to the pulsation valve (35).



Switch off the pulsation valve motor (34). Close the air pressure line needle valve (29). Turn off the steel gas cylinder (58) valve.

*Computation of Reservoir Chamber Pressure.*—The pulsating pressure in the reservoir chamber (18) must be set above the pressure desired on the organ, an amount equal to the pressure required to lift the column of perfusion fluid in the feed tube (20) to the cannula (3), plus the back pressure generated in the organ chamber during operation.

*Constant Pressure.*—A comparatively constant pressure can be exerted on the organ, if desired, by closing the clamp (45) governing the constricted opening which bypasses the one-way gas valve (44).

*Floating the Organ.*—The organ can be floated in the perfusion fluid by removing the pinch-cock (50) from the rubber tube on the organ chamber inner filter bulb (2), and allowing the fluid level to rise in the organ chamber until the organ is covered, before replacing the pinch-cock (50). A twisted artery can sometimes be straightened out this way.

*Stopping Pulsation Valve.*—If it is necessary to stop the pulsation valve (35), temporarily, in order to adjust some part of the pulsation system, the following procedure is satisfactory. Gradually shut the clamp (37) on the air pressure line leading to the pulsation valve (35). Switch off the pulsation valve motor (34). Make the adjustment desired. Switch on the pulsation valve motor (34). Gradually open the clamp (37) on the air pressure line leading to the pulsation valve (35), until the desired pressure is reached on the organ. While opening this clamp (37), care must be taken that the oil level in the oil flask inner chamber (25) does not overflow and close the oil check valve (41). If necessary, let additional control gas into the oil flask inner chamber (25) to prevent overflow. Open clamp on the control gas line and let the control gas flow into the oil flask inner chamber (25), until the gas starts to bubble into the oil flask outer chamber (27). Then adjust the flow of control gas to the rate desired.

*Filming in Reservoir Chamber.*—Bubbling and filming in the main reservoir chamber (18) are caused by lack of proper adjustment of the fluid level in the equalization chamber (13), and may be stopped by raising the fluid in the equalization chamber to the proper level.

*Collapse of Artery.*—A collapse of the artery, leading from the cannula to the organ, during the release portion of the pulsation cycle, is caused if the clamp (48) is closed on the gas line leading to the equalization chamber (13). Adjustment of the clamp (48) will remedy this situation.

*Pulsation Pressures after Installation of Perfusion Pump.*—The pulsation pressures will not be properly transmitted to the organ when the pump is first installed in the incubator, unless there is sufficient fluid in the upper floating valve reservoir (14) to float the valve (9). This reservoir (14) should be filled (before the organ is attached to the cannula (3), and after the perfusion fluid has been inserted in the pump) by connecting a rubber tube to the reservoir chamber filter bulb (22), and blowing through the tube until sufficient perfusion fluid has passed through the cannula (3) to wet the sand filter (6) and fill the upper floating valve reservoir (14).

*Fluid Rise in Organ Chamber.*—If the perfusion fluid level rises in the organ chamber, it is an indication of a leak past one of the glass and rubber connections, or past the pinch-cock (50) closing the inner filter bulb (2) rubber tube. The organ chamber should be opened to atmospheric pressure, and another coat of waterproof cement applied to all joints between glass and rubber. The rubber tube leading to the inner filter bulb (2) should be coated with vaseline on the inner surface where it is closed by the pinch-cock (50).

*Overflow of Oil Flask.*—Any large leak of the control gas will cause the oil to overflow the oil flask inner chamber (25) and close the oil check valve (41). If this happens, the pulsating pressure in the gas line to the pulsation pumps is shut off and the circulation of perfusion fluid ceases.

The circulation should be started again by the following procedure. Close the clamp (37) on the air pressure line leading to the pulsation valve (35). Open the clamp (39) on the control gas line leading to the oil flask inner chamber (25), until the gas bubbles into the outer chamber (27). Then close the clamp (39). The pulsation valve (35) should be running while the control gas is being let into the inner chamber (25), so that the replaced air in the outer chamber (27) may escape. Disconnect the oil check valve (41) and all parts of the gas line which contain oil. Remove the oil and reconnect. Repair the leak which caused the overflow. If much oil has been lost, refill the oil flask to the proper level. Gradually open the clamp (37) on the air pressure line leading to the pulsation valve (35), until the desired pulsating pressure is reached. Care must be taken that oil does not again overflow the oil flask inner chamber while the clamp (37) is being opened. If the oil level becomes too high in the inner chamber (25), more control gas should be let into the chamber. Adjust the flow of control gas into the oil flask inner chamber (25).

*Inspection of Rubber Tubing.*—Oil and grease will cause rubber tubing to swell in time. Inspection of rubber tubing should be made at intervals to make sure that it is still strong and that its inner diameter is not obstructed. This is particularly necessary in regard to rubber tubing leading to and from the pulsation valve (35).

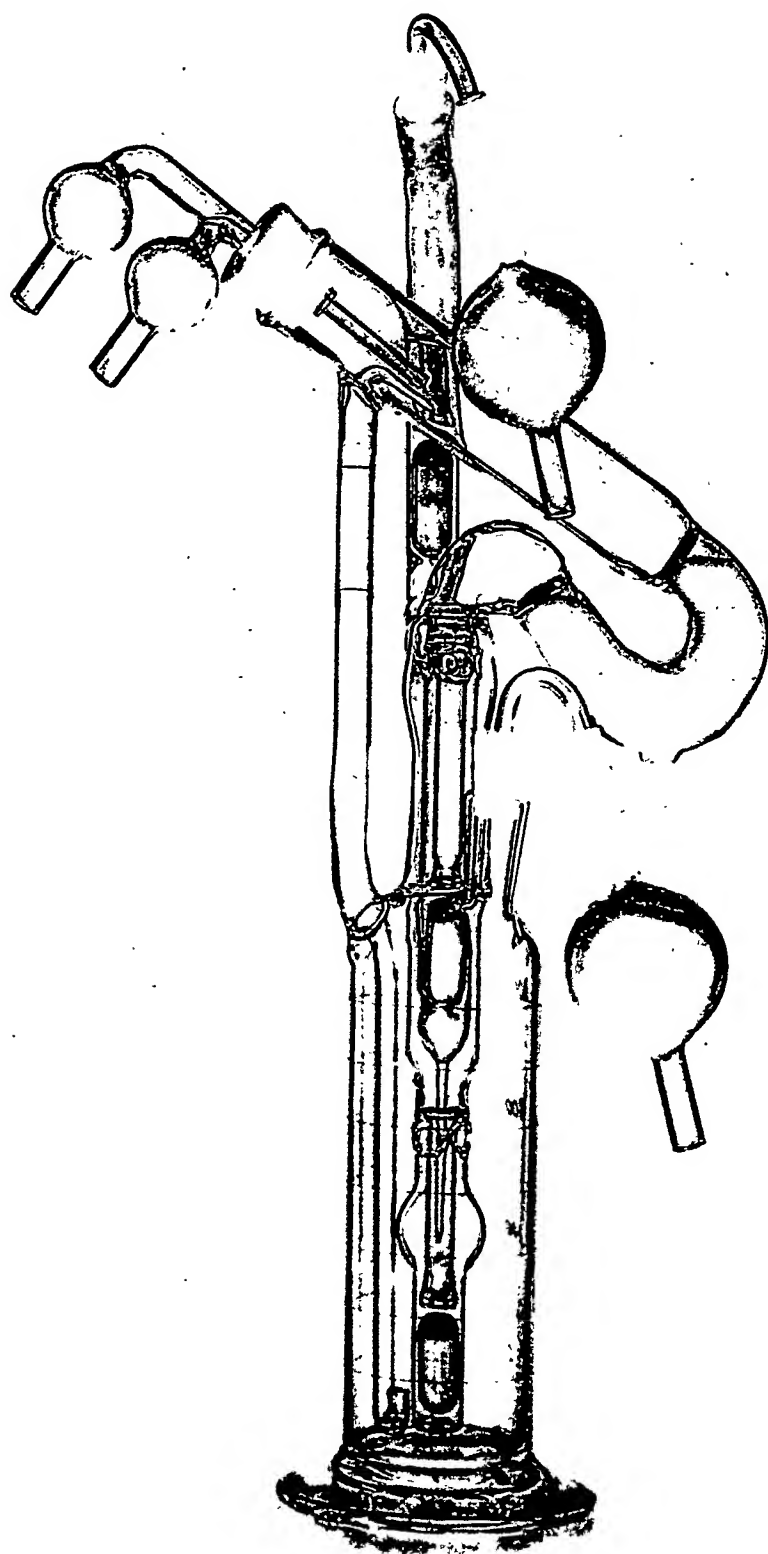
#### SUMMARY

An apparatus has been developed which maintains, under controllable conditions, a pulsating circulation of sterile fluid through organs for a length of time limited only by the changes in the organ and in the perfusion fluid.

#### EXPLANATION OF PLATE 15

Fig. 1. Perfusion pump.





Photographed by Louis Schmitt and Joseph B. Haulenberg.

FIG. 1

Van der Pijp. Apparatus for culture of white mouse.



## RIFT VALLEY FEVER

### A REPORT OF THREE CASES OF LABORATORY INFECTION AND THE EXPERIMENTAL TRANSMISSION OF THE DISEASE TO FERRETS

By THOMAS FRANCIS, JR., M.D., AND T. P. MAGILL, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research)*

PLATES 16 AND 17

(Received for publication, June 6, 1935)

Rift Valley fever, a disease of sheep and cattle, was first studied and described in the Kenya Colony, British East Africa, by Daubney, Hudson, and Garnham (1), who showed that the specific causative agent of the disease is a filterable virus. Findlay and Daubney (2) found that the virus is infectious for a wide variety of animal hosts, and especially for rodents. That the virus is infectious for man as well, was noted by Daubney, Hudson, and Garnham (1), who reported the prevalence of the disease among sheep herders during the epizootic. Moreover, these investigators transmitted the disease directly to man by the intramuscular inoculation of the blood of infected sheep. The disease in man has been described as dengue-like (1), or influenza-like (3), and is characterized by sudden onset, fever, and severe pains in the back and extremities.

Further evidence of the infectiousness of the virus for man has been obtained from the high incidence of the disease among laboratory workers engaged in studying the experimental disease. Kitchen (4) has recently summarized thirteen cases of Rift Valley fever of laboratory origin. In each instance the diagnosis was based upon the recovery of the virus from the blood of the patient during the acute illness, the demonstration of specific neutralizing antibodies in the convalescent serum, or both. The presence of the virus of Rift Valley fever in human blood is most readily demonstrated by the inoculation of the blood into mice, in which a rapidly fatal disease of a septicemic nature is produced (5). Marked hepatic necrosis is an outstanding feature of the infection in mice, and acidophilic intranuclear inclusion bodies are found in great numbers in the damaged liver cells.

The actual mode of infection of the human individual with Rift Valley fever has not been established, although Findlay (5) suggests the possibility that infection



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The actual mode of infection of the human individual with Rift Valley fever has not been established, although Findlay (5) suggests the possibility that infection



occurs through an absorption of the virus through the conjunctival sac or through the nasal mucous membranes. Furthermore, by the injection of Pitt Valley fever virus into the nostrils of monkeys, he induced a febrile disease in those animals similar to that occurring spontaneously in man.

All the previously reported cases of laboratory infection occurred during a period of active study of the virus, so that the diagnosis of Pitt Valley fever was readily suspected. The present report deals with three cases of Pitt Valley fever in human individuals, in the first of which the source of the infection is obscure. The disease in the other two cases appears to have been related directly to the handling of infectious material. The virus was shown to be present in the upper respiratory tracts of two of the individuals, as demonstrated by the intranasal inoculation of ferrets with nasopharyngeal washings of both patients. In these ferrets a disease was produced in which pulmonary involvement was the outstanding feature.

### *Report of Cases*

*Case 1, E. H.*—The patient, a male laboratory assistant, during the early morning of Oct. 6, 1934, felt chilly and experienced generalized aching. He was seen that afternoon by a physician, who noted no abnormal physical findings except mild injection of the throat. His temperature by mouth was 103°F. On the following day, after antipyretics, the temperature was 99.5°, and the next day was normal. The aches and pains were less severe. Recovery was uneventful except that the patient complained for some time of shooting pains in his muscles.

Because of the similarity of the clinical symptoms to those of influenza, pharyngeal washings obtained on the 2nd day of illness were inoculated intranasally into a ferret. The ferret became ill 2 days later, and with material obtained from this ferret, sacrificed on the 4th day of illness, it was possible to transmit the disease to other ferrets.

*Case 2, T. F.*—The patient, a member of the staff, working with the virus obtained from the previous case, was suddenly awakened at 2 a.m. Nov. 10, 1934, with severe chills. During the day his temperature rose to 104.5°, and was accompanied by headache, generalized bodily pains, moderate nasal congestion, tenderness of the eyeballs, and mental confusion. The leukocyte count in the morning was 4880; in the afternoon 3720. The urine showed no abnormalities. The patient was considered to be suffering from a typical attack of influenza. The following day, after the administration of antipyretics, the fever was lower, but

marked backache persisted. The leukocyte count was 4780 with 48 per cent polymorphonuclear leukocytes. On the 3rd day the patient's temperature returned to normal and remained so. Convalescence proceeded rapidly without complications. There persisted for some time, however, pain on motion of the eyes, and a sense of imbalance.

Throat washings obtained from the patient on the 1st day of illness were inoculated into the nasal passages of a ferret. The animal became ill, and the course of the infection was similar to that of the ferret inoculated with the washings from the previous case. Pharyngeal washings obtained from the patient on the 7th day after the onset of infection failed to produce the disease in a ferret. The blood of this patient, taken on the 1st day of illness, was also found to contain active virus.

TABLE I  
*Recovery of Virus from Human Cases of Rift Valley Fever*

| Case No.                      | Material from which virus was isolated |          |
|-------------------------------|--|----------|
|                               | Throat washings                        | Blood    |
| 1. E. H., Oct. 6, 1934. ....  | +                                      | Not done |
| 2. T. F., Nov. 10, 1934. .... | +                                      | +        |
| 3. S. S., Dec. 12, 1934. .... | -                                      | +        |

+ = virus recovered from the indicated material.

- = virus not recovered from the indicated material.

*Case 3, S. S.*—The patient, a male laboratory assistant, actively engaged in the studies of the two previous strains of virus, was taken sick suddenly on Dec. 12, 1934. The onset was marked by a severe chill, at which time his temperature was 99.8°F. by mouth. There was a slight cough. His throat was red and a throat culture taken at this time revealed a heavy growth of hemolytic *Hemophilus influenzae*. The leukocyte count was 9700. During the night his temperature was reported to have risen to 105.6°F. There was sleeplessness, some epistaxis, nausea and vomiting, a distinct sense of anxiety, and generalized aches and pains. The latter, the patient described as not being as severe as those of influenza which he had experienced 3 months previously. He was admitted to the hospital on Dec. 13, 1934, with a temperature of 103°F. and white blood cell count of 5650 with 80 per cent polymorphonuclears. The urine showed no abnormalities. There was a mild but persistent nosebleed. The patient's temperature fell rapidly and he apparently had recovered completely when discharged from the hospital on the 8th day after admission. 2 days later he again developed fever, severe

headache, nausea and some vomiting, and experienced dizziness for several days. Recovery proceeded uninterruptedly thereafter.

The pharyngeal washings taken from the patient at the onset of the illness did not contain demonstrable virus, but the blood obtained on the 2nd day of illness was found to be infectious for mice.

The course of the disease in this individual resembles the previously reported cases of Rift Valley fever with febrile relapses (1, 5). In none of the present cases was there any evidence of visceral damage. In fact, the clinical diagnosis of influenza was made unhesitatingly in each instance.

### *The Transmission of Rift Valley Fever to Ferrets*

The pharyngeal washings of Cases 1 and 2 were inoculated into the nasal passages of ferrets anesthetized with ether. The subsequent course of events in both instances was so similar that they can be considered together. After an interval of 48 hours, the temperature of the ferrets rose abruptly to 106°F., the animals became apathetic, displayed no interest in food, and respirations were rapid. The course of the temperature is shown in Chart 1. The animals were sacrificed on the 4th day of fever. At autopsy, a bluish edematous consolidation of the lower lobe of the left lung was observed. The liver was pale and rather brown. There was distinct hyperemia of the lower two-thirds of the intestines. The spleen was somewhat enlarged. The adrenal glands and kidneys showed no gross abnormalities.

With the intranasal inoculation of suspensions of finely ground lung tissue from an infected ferret, or with Berkefeld filtrates of such suspensions, it was possible to transmit the disease serially in these animals. With successive transfers the infection became more severe, and when allowed to continue was generally fatal. The pulmonary lesions became more extensive so as to involve almost the entire lung. Copious amounts of blood-stained mucus collected in the trachea. At times hemorrhagic diarrhea occurred and dark, changed blood was found in the intestines. On one or two occasions gross hemorrhages were noted about the adrenal glands. The spleen was usually enlarged. The liver was light brown, and in some instances gross areas of necrosis were visible to the naked eye.

It was subsequently found that the subcutaneous injection of

emulsions of infected ferret lung into normal ferrets also caused death; in the two instances observed, pulmonary lesions were present even after subcutaneous inoculations.

*Pathology of Rift Valley Fever in the Ferret*

In describing the pathological changes which were observed in infected ferrets, it must be borne in mind that the majority of the animals were sacrificed on the 4th or 5th day of illness, and that the size of the infecting dose of virus was progressively decreased. The

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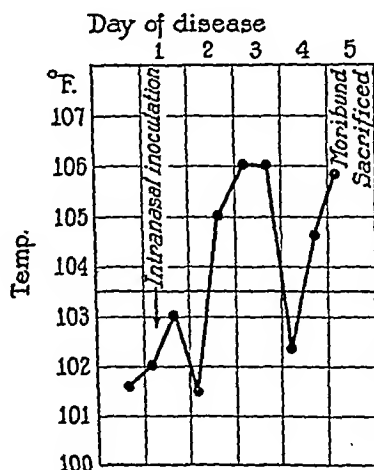


CHART 1. Temperature chart of a ferret infected with Rift Valley fever virus.

most severe intestinal and hepatic lesions were observed in the animals of the early passages.

*Lungs.*—The lungs of ferrets which die or are sacrificed after intranasal inoculation present in the gross a massive bluish gray consolidation of lobar distribution. The consolidation usually involves all of the basal lobes, the cardiac and right middle lobes, and, less completely, the upper lobes. When the infected lung is cut, a large amount of thin serous fluid exudes, and the size of the lobe decreases considerably. Cultures of the involved lungs are almost invariably bacteria-free.

Microscopically, the outstanding features of the pneumonia are a moderate edema and cellular proliferation in the alveolar walls, the great amount of edema fluid and the comparatively scanty cellular exudate in the alveoli. The cells comprising the exudate are primarily of the large acidophilic mononuclear variety

similar to those seen in the lungs of ferrets infected with influenza virus (7, 8). Inclusion bodies were not observed. No noteworthy changes were seen in the walls of the bronchi or bronchioles, although a moderate amount of exudate, containing polymorphonuclear leukocytes, was not infrequently present in the lumen. The peribronchial lymphatic channels are, at times, quite dilated. Vascular hyperemia is usually noted. The walls of the blood vessels may be very edematous, the cells of the endothelium are swollen, and a moderate perivascular round cell infiltration is commonly present. (Fig. 1.)

*Liver.*—The lesions in the liver are of irregular severity. At times the entire organ is light brown in color with nodular areas of necrosis 0.5 cm. in diameter. At other times there is little gross change except increased friability. The liver is usually swollen. Microscopic study, in the majority of instances, revealed very mild, if any, damage to the liver. On the other hand, in the livers of the early passage ferrets, marked hemorrhagic necrosis of zonal distribution was seen. At other times all the hepatic cells appeared to be undergoing fatty degenerative changes. The nuclei were distinct, but no inclusion bodies were seen. Some infiltration of small mononuclear cells about the portal radicals was observed. The Kupffer cells were not obviously affected. (Figs. 3 and 4.)

*Spleen.*—The spleen is usually enlarged and exhibits active hematopoiesis. Apart from moderate hyperemia or edema, significant changes were not observed.

*Intestines.*—In the gross, hyperemia of most of the large intestine is frequently present, and at times either fresh blood or blackened, changed blood may be found in the lumen. No gross ulcerations were observed. Microscopically, in the majority of instances, little of significance is seen except hyperemia of the capillaries of the villi. In one instance, in which fresh blood was present in the intestine, the sections revealed distinct hemorrhage in the villi, and, in some areas, desquamation of the lining epithelium. These changes resemble those described in the intestinal canal of infected sheep (1, 5). (Fig. 2.)

*Adrenals.*—The adrenals were examined only in those instances in which gross hemorrhage was observed. The hemorrhage was limited to the subcapsular tissue, while the glandular structure was entirely normal.

*Kidneys.*—The kidneys usually show no gross changes other than cloudy swelling. In one animal a few small hemorrhages were noted in the cortex. Microscopically, in this case, marked congestion of the glomerular capillaries and intertubular vessels was seen. Albuminous exudate was present in the tubules and in the glomerular spaces.

The most consistent pathological changes, apart from those in the lungs of the ferrets, were the hemorrhagic extravasations in different organs. These lesions are apparently related to an increased permeability of the blood vessels or to marked congestion rather than to destructive changes in the vascular walls. In contrast to the disease in sheep and mice, the extensive, almost complete hepatic necrosis is

not commonly seen in ferrets. Furthermore, in sections of the ferret organs, acidophilic intranuclear inclusion bodies have not been observed.

### *Rift Valley Fever in Mice*

When bacteria-free suspensions of lung from the infected ferrets were inoculated into the nasal passages of anesthetized mice, death of the mice occurred in 3-4 days. Pulmonary lesions were not observed, but the livers of the mice so infected presented the extensive necrosis and acidophilic intranuclear inclusions characteristic of Rift Valley fever. It was possible to continue to transmit the infection to normal mice by the intranasal, intraperitoneal, subcutaneous, or intracerebral inoculation of the blood, or suspensions of lung or liver from infected mice.

Furthermore, serum of the second and third patients taken at the height of the disease was found to contain active virus which produced the characteristic disease in mice. After standing in the ice box 10 weeks, the serum still contained considerable virus. On the other hand, the direct intranasal inoculation of mice with washings from the throats of the patients did not induce Rift Valley fever in these animals.

### *Identification of the Virus*

It was found that convalescent serum from Patients 1 and 2 reciprocally neutralized in ferrets the activity of the strains of virus recovered from the throats of these two patients. The ferrets receiving mixtures of convalescent serum and active virus developed neither fever nor pulmonary lesions.

The serum of ferrets after recovery from the disease was found to contain antibodies which specifically neutralized the infectivity of the virus. Nevertheless, two of these animals, reinoculated intranasally after an interval of 2 months, again became sick with fever and respiratory symptoms.

Neutralization tests were also carried out in mice. The livers of mice infected with the E. H. strain were used as the source of virus. Weighed amounts of liver were ground with 10 per cent normal rabbit serum in distilled water. After centrifugation at high speed for 15 minutes the material was further diluted serially to

make  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  concentrations, in terms of the original liver weight. In dilutions as high as 1:10 million, 0.2 cc. of the material injected into the peritoneal cavity of a mouse caused death in 2-3 days. 0.5 cc. portions of the respective dilutions were mixed with 1.0 cc. amounts of the serum to be tested and incubated at 37°C. for 30 minutes. Each of a group of three mice was then inoculated intraperitoneally with 0.4 cc. of the serum-virus mixture.

TABLE II  
*Identification of Virus as Rift Valley Fever by Neutralization Tests in Mice*

| Human sera                              | Dilution of virus suspension |    |    |           |    |    |           |    |    |           |    |    |
|---|------------------------------|----|----|-----------|----|----|-----------|----|----|-----------|----|----|
|   | $10^{-3}$                    |    |    | $10^{-4}$ |    |    | $10^{-5}$ |    |    | $10^{-6}$ |    |    |
| Normal                                  |                              |    |    |           |    |    |           |    |    |           |    |    |
| E. M.....                               | D2                           | D2 | D2 | D2        | D2 | D2 | D2        | D2 | D2 | D2        | D2 | D2 |
| R. P.*.....                             | D2                           | D3 | D3 | D3        | D3 | D3 | D3        | D3 | D3 |           |    |    |
| T. M.*.....                             | D2                           | D2 | D3 | D2        | D2 | D2 | D2        | D2 | D2 |           |    |    |
| M. T.*.....                             | D2                           | D2 | D3 | D2        | D2 | D2 | D2        | D2 | D2 |           |    |    |
| Convalescent                            |                              |    |    |           |    |    |           |    |    |           |    |    |
| 1. E. H.....                            | S                            | S  | S  | S         | S  | S  | S         | S  | S  |           |    |    |
| 2. T. F.....                            | S                            | S  | S  | S         | S  | S  | S         | S  | S  |           |    |    |
| 3. S. S.....                            | S                            | S  | S  | S         | S  | S  | S         | S  | S  |           |    |    |
| Known Rift Valley immune<br>(N.O.)..... | S                            | S  | S  | S         | S  | S  | S         | S  | S  |           |    |    |
| Animal sera                             |                              |    |    |           |    |    |           |    |    |           |    |    |
| Normal ferret.....                      | D2                           | D2 | D2 | D2        | D2 | D2 | D2        | D2 | D2 | D3        | D3 | D3 |
| Convalescent ferret                     |                              |    |    |           |    |    |           |    |    |           |    |    |
| Rift Valley fever virus                 |                              |    |    |           |    |    |           |    |    |           |    |    |
| Strain 1.....                           | S                            | S  | S  | S         | S  | S  | S         | S  | S  |           |    |    |
| Strain 2.....                           | S                            | S  | S  | S         | S  | S  | S         | S  | S  |           |    |    |
| Influenza virus                         |                              |    |    |           |    |    |           |    |    |           |    |    |
| Strain P. R. 5.....                     | D2                           | D2 | D3 | D2        | D2 | D2 | D2        | D2 | D2 |           |    |    |
| Strain P. R. 8.....                     | D2                           | D2 | D2 | D2        | D2 | D2 | D1        | D2 | D2 |           |    |    |
| Known Rift Valley immune<br>monkey..... | S                            | S  | S  | S         | S  | S  | S         | S  | S  |           |    |    |

D = died. Numerals indicate day after infection on which death occurred.  
 S = survived without evidence of infection.  
 Strains 1 and 2 are those recovered from Case 1 (E.H.) and Case 2 (T.F.) respectively.  
 Strains P.R. 5 and P.R. 8 are strains of human influenza virus obtained from influenza patients in Puerto Rico.  
 \* Normal individuals who had been exposed to infection, but had experienced no illness.

It was found that the convalescent sera of all three patients, and the serum from recovered ferrets, protected mice against at least 1000 fatal doses of the virus. The serum of a human individual previously shown to neutralize Rift Valley fever virus, and the serum of a monkey immunized<sup>1</sup> against Rift Valley fever virus, were also found to afford complete protection to mice against the virus recovered from the three cases of human infection reported in the present study.

The serum of normal men and of three individuals who had been exposed to infection with the virus contained no neutralizing antibodies. The serum of ferrets recovered from infection with the virus of influenza likewise failed to protect mice against infection with the Rift Valley fever virus.

The evidence which led to the conclusion that the disease which had been encountered was Rift Valley fever may be briefly summarized: The virus obtained from the pharyngeal washings or from the blood of the patients induced an experimental disease in mice which was identical with that produced in these animals by the virus of Rift Valley fever. The infectivity of the virus was neutralized not only by the serum of the convalescent patients and of ferrets which had recovered from the experimental disease, but also by the serum of a human individual who was known to have had Rift Valley fever, and by the serum of a monkey which had been immunized with the virus of Rift Valley fever. Both sera were known to contain neutralizing antibodies for Rift Valley fever virus. From the experimental evidence, therefore, it may be concluded that the virus recovered from the three patients reported in this paper is the virus of Rift Valley fever.

#### *Differential Diagnosis of Rift Valley Fever and Influenza*

The difficulty of differentiating Rift Valley fever from influenza on purely clinical grounds is emphasized in the cases here reported. In only the third case was the diagnosis of influenza questioned, and, in this case, only because the patient had experienced a proven attack of influenza but 3 months previously. A second attack of the same infection within so short a time seemed unlikely. Nevertheless, there was no distinct clinical feature which served to differentiate one infec-

<sup>1</sup> The monkey was immunized by Dr. T. M. Rivers.



tion from the other. The blood of this patient taken during the acute stage of his second illness was found to cause a fatal, non-bacterial disease in mice. Since results of this nature have not been obtained with the blood of influenza patients, it was suggested that the two illnesses were unrelated and of different etiology. Chart 2 presents the temperature curves of the patient during the attack of influenza and during the later illness which was shown to be Rift Valley fever.

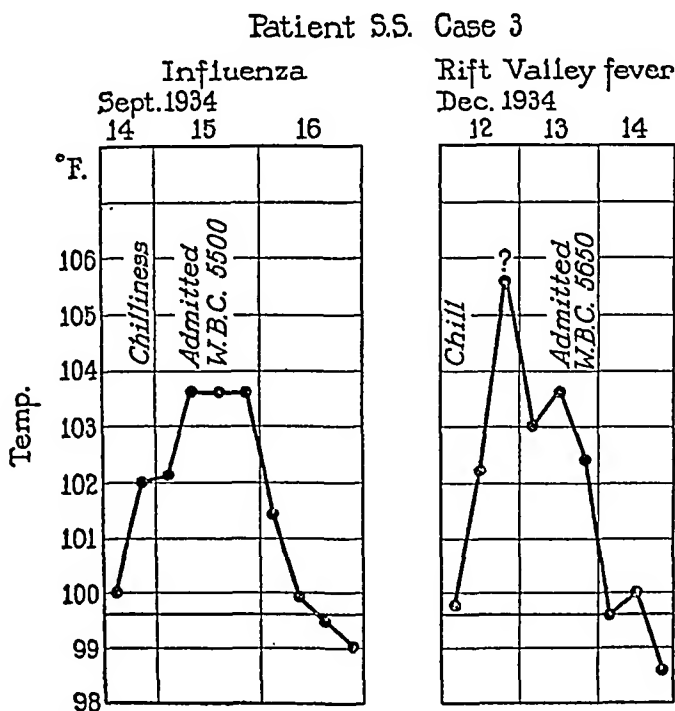


CHART 2. Temperature charts of the same patient (S. S.) during an attack of influenza and a subsequent attack of Rift Valley fever.

A comparison of the two curves again illustrates the close similarity of the clinical course in the two diseases.

The susceptibility of ferrets to infection with the virus of Rift Valley fever has not previously been reported. The susceptibility of these animals to the virus of influenza (6) and the pulmonary involvement which follows the intranasal inoculation of human (7) or swine (8) influenza virus was known. The febrile reaction and the pulmonary lesions produced by Rift Valley fever virus in ferrets differ only in

severity from those caused by the viruses of human and swine influenza. The virus of Rift Valley fever is infective for ferrets by the subcutaneous, as well as by the intranasal route, and produces lesions in organs other than the lungs. These facts serve to differentiate it from the virus of influenza.

TABLE III  
*Differential Diagnosis of Rift Valley Fever and Influenza*

|                                | Influenza                                 | Rift Valley fever                      |
|--------------------------------|---|--|
| Man                            |   |  |
| Onset                          | Abrupt with chills                        | Same                                   |
| Symptoms                       | Generalized aches, etc., mild respiratory | Same                                   |
| Course                         | Short and sharp                           |  |
| Complications                  | Usually none                              | Same                                   |
| W. B. C.                       | Leukopenia                                | Same                                   |
| Virus in                       | Respiratory tract                         | Same                                   |
| Ferret                         |   | Respiratory tract and blood            |
| Incubation                     | 24 to 72 hrs.                             | Same                                   |
| Lung lesions                   | Only after several passages               | First passage. More extensive          |
| Liver lesions                  | Cloudy swelling                           | Focal necrosis. At times very marked   |
| Intestinal lesions             | None                                      | Hemorrhagic enteritis                  |
| Outcome                        | Recovery                                  | Usually death                          |
| Mouse                          |   |  |
| Lung lesions                   | Marked                                    | None                                   |
| Liver lesions                  | None                                      | Marked. Acidophilic nuclear inclusions |
| Virus in                       | Lungs                                     | Blood, liver, lungs, others            |
| Effective route of inoculation | Intranasal                                | All routes                             |

The results of mouse inoculation, however, afforded the most striking evidence of the differences in the two diseases. In mice, the disease produced by the virus of influenza is apparently limited to involvement of the respiratory tract, with bluish red pulmonary consolidation. Rift Valley fever virus is infectious in high titer for mice by all routes, and invades the blood and all other organs. Pulmonary lesions were not observed even after intranasal infection. Extensive hepatic

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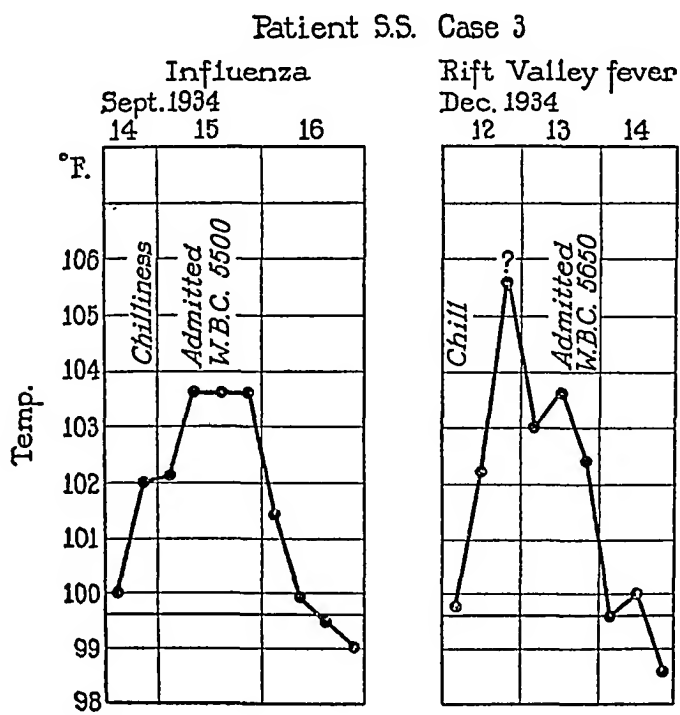


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| Course                         | Short and sharp                           | Same                                   |
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| Virus in                       | Respiratory tract                         | Respiratory tract and blood            |
| Ferret                         |   |  |
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| Intestinal lesions             | None                                      | Hemorrhagic enteritis                  |
| Outcome                        | Recovery                                  | Usually death                          |
| Mouse                          |   |  |
| Lung lesions                   | Marked                                    | None                                   |
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| Virus in                       | Lungs                                     | Blood, liver, lungs, others            |
| Effective route of inoculation | Intranasal                                | All routes                             |

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necrosis, with acidophilic intranuclear inclusions, occurs no matter by what route the virus is introduced.

### *Immunization of Rabbits*

Although they considered the rabbit an insusceptible host, Findlay and Daubney (2) reported that the virus of Rift Valley fever could be demonstrated in the blood of these animals for several days after intravenous administration. No reference is made, however, to the development of specific immune bodies in the serum of rabbits which were inoculated with the virus.

In the present study, large doses of the suspensions of lung of infected ferrets were inoculated into rabbits intracerebrally, intracutaneously, and intraperitoneally. In no case did the animal become sick or even exhibit a febrile reaction. In spite of the lack of evidence of active infection, it was of interest to determine whether neutralizing antibodies developed in the serum of rabbits inoculated with virus-containing material. To test this possibility, rabbits were given two intraperitoneal injections 4 weeks apart, of suspensions of lungs of infected ferrets, and were bled 15 days after the last injection. The serum of these rabbits was found to contain a high titer of neutralizing antibodies for Rift Valley fever virus as measured by the mouse protection test. The results are similar to those reported by Whitman (9) with the virus of yellow fever.

### *The Effect of the Route of Administration of Serum-Virus Mixtures upon the Protective Action of Immune Serum*

When the neutralization test in mice is performed by the intraperitoneal inoculation of mixtures of serum and Rift Valley fever virus, the results are usually quite sharp. All mice receiving normal serum and virus die in 2-3 days, while the mice injected with mixtures of virus and immune serum survive without evidence of infection.

If the mixtures are given by the intranasal route to mice under ether anesthesia, certain sera fully effective by the intraperitoneal route may exhibit little or no protective action. In other instances, after 8-10 days, irregular deaths may occur among the mice receiving mixtures of immune serum and virus, suggesting that during the interval the neutralizing action of the serum has been dissipated and

has merely delayed the fatal outcome. From the animals which succumb to the delayed infection, fully active virus may be recovered. In Table IV are presented the results of comparative tests done by the intranasal and intraperitoneal routes.

These results indicate that the protective action of immune serum is not exerted directly upon the virus, since the period of exposure of virus to immune serum *in vitro* was the same in both series of tests. The neutralizing efficiency of the immune serum appears rather to be influenced by the route by which the serum-virus mixture is administered.

TABLE IV

*The Influence of the Route of Administration upon the Neutralizing Capacity of Serum*

| Serum               | Route of inoculation | Dilutions of virus |    |    |                  |    |    |                  |    |    |
|---------------------|----------------------|--------------------|----|----|------------------|----|----|------------------|----|----|
|                     |                      | 10 <sup>-3</sup>   |    |    | 10 <sup>-4</sup> |    |    | 10 <sup>-5</sup> |    |    |
| Normal human        | i.n.                 | D3                 | D3 | D3 | D3               | D3 | D3 | D4               | D4 | S  |
|                     | i.p.                 | D2                 | D2 | D2 | D2               | D2 | D2 | D2               | D2 | D2 |
| Convalescent human  | i.n.                 | D3                 | D3 | D6 | D4               | D4 | D4 | D5               | S  | S  |
|                     | i.p.                 | S                  | S  | S  | S                | S  | S  | S                | S  | S  |
| Normal ferret       | i.n.                 | D2                 | D3 | D3 | D2               | D3 | D3 | D4               | D4 | D4 |
|                     | i.p.                 | D2                 | D2 | D2 | D2               | D2 | D2 | D2               | D2 | D2 |
| Convalescent ferret | i.n.                 | D8                 | S  | S  | D8               | D9 | S  | S                | S  | S  |
|                     | i.p.                 | S                  | S  | S  | S                | S  | S  | S                | S  | S  |

i.n. = intranasal.

i.p. = intraperitoneal.

D = died. Numerals indicate day after infection on which death occurred.

S = survived.

#### DISCUSSION

The unusual feature in this outbreak of Rift Valley fever in man is that the source of infection of the first case (E.H.) was not definitely established. No Rift Valley fever virus had been used for study in these laboratories for 4 months prior to the onset of the illness. Furthermore, this patient had never assisted with the actual investigative work, nor had he ever been exposed to animals used in the study. The mice which had been employed were constantly kept in a room



An interesting observation was made regarding the influence of the route of administration upon the specific protective action of immune serum in mice. When given alone by the intranasal or intraperitoneal route, the virus of Rift Valley fever invades the blood of mice and profoundly damages the liver. When given together with immune serum by the intraperitoneal route, no evidence of infection is observed, but when immune serum and virus are administered by the intranasal route there may be little detectable difference between the protective effects of immune and normal serum. The difference in the protective action of the same serum administered by two different routes must, therefore, be related to a difference in the responses of the tissues into which the serum-virus mixtures are introduced.

These results offer support to the views of other investigators who have indicated that immune serum does not act directly upon the virus with a virucidal effect (11-13). Furthermore, the results suggest that the reaction of the tissue which first encounters the serum-virus mixture may be an important factor in determining the neutralizing efficiency of immune serum. A somewhat similar concept has been discussed recently by Sabin (13).

In the presence of an epidemic of influenza, it may be difficult clinically to differentiate Rift Valley fever from influenza. In the ferret the gross aspects of the experimental disease differ from those of influenza only in their severity. The chief aid in differential diagnosis is the presence of the virus of Rift Valley fever in the circulating blood of the patient during the acute illness. The virus can be recovered by the inoculation of the blood into mice, in which animals characteristic pathological lesions are produced.

#### SUMMARY

Three cases of Rift Valley fever in human individuals are reported. The virus was recovered from the respiratory tract of the patients and was transmitted to ferrets by the intranasal route. The experimental disease so produced in ferrets is characterized by fever, marked pulmonary lesions, and hemorrhagic phenomena. The results indicate that the virus of Rift Valley fever belongs to the group of filterable viruses which may gain entrance to the human body through the respiratory tract.



under strict quarantine. The jars in which they were placed stood in pans of lysol, and the legs of the table on which the jars rested stood in lysol.

3 months after all animals had been removed from the room, the patient assisted in scraping and painting the walls and floor. So far as can be determined, that task was completed 15 days before the onset of the disease. If the infection in the present instance was acquired by exposure to virus persisting in this room, the virus must have withstood very adverse conditions for over 3 months, remaining not only viable but infectious. Similarly, the incubation period of the resultant infection, if thus acquired, was considerably prolonged, since all other records indicate that the period of incubation in Rift Valley fever in man is 6 days or less (5).

Another interesting aspect of the study of the human cases of the disease is that the virus of Rift Valley fever was readily recovered from the upper respiratory tract of two of the patients during the first 3 days of the disease, whereas on the 8th day after onset the virus could not be demonstrated in the throat washings of the one individual studied (Case 2). These observations clearly indicate that the virus of Rift Valley fever belongs to the group of filterable viruses which may effect their entry to the human body through the respiratory tract. Further evidence of the capacity of the virus to invade by way of the respiratory tract was obtained by the inoculation of the virus into the nasal passages of ferrets. The experimental disease in the ferret is characterized by the development of extensive edematous pulmonary consolidation with a scanty exudate of large mononuclear cells. The pulmonary lesion resembles that produced in experimental animals by other virus diseases, for example influenza (7, 8) and psittacosis (10).

In addition to the pathological features of the disease in mice, which were identical with those of Rift Valley fever, the virus recovered from patients in the present study was identified as that of Rift Valley fever by means of neutralization tests in mice. Not only was the infectivity of the virus neutralized by the serum of all three patients during convalescence, and by the serum of recovered ferrets, but by known Rift Valley fever immune serum as well. It was therefore established that the disease was Rift Valley fever.

An interesting observation was made regarding the influence of the route of administration upon the specific protective action of immune serum in mice. When given alone by the intranasal or intraperitoneal route, the virus of Rift Valley fever invades the blood of mice and profoundly damages the liver. When given together with immune serum by the intraperitoneal route, no evidence of infection is observed, but when immune serum and virus are administered by the intranasal route there may be little detectable difference between the protective effects of immune and normal serum. The difference in the protective action of the same serum administered by two different routes must, therefore, be related to a difference in the responses of the tissues into which the serum-virus mixtures are introduced.

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The differential diagnosis of Rift Valley fever and influenza is discussed. While, clinically, this is a difficult problem, the diagnosis may be readily established through animal experimentation.

Certain observations concerning the influence of the route of administration on the protective action of immune serum in serum-virus mixtures are presented.

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#### EXPLANATION OF PLATES

##### PLATE 16

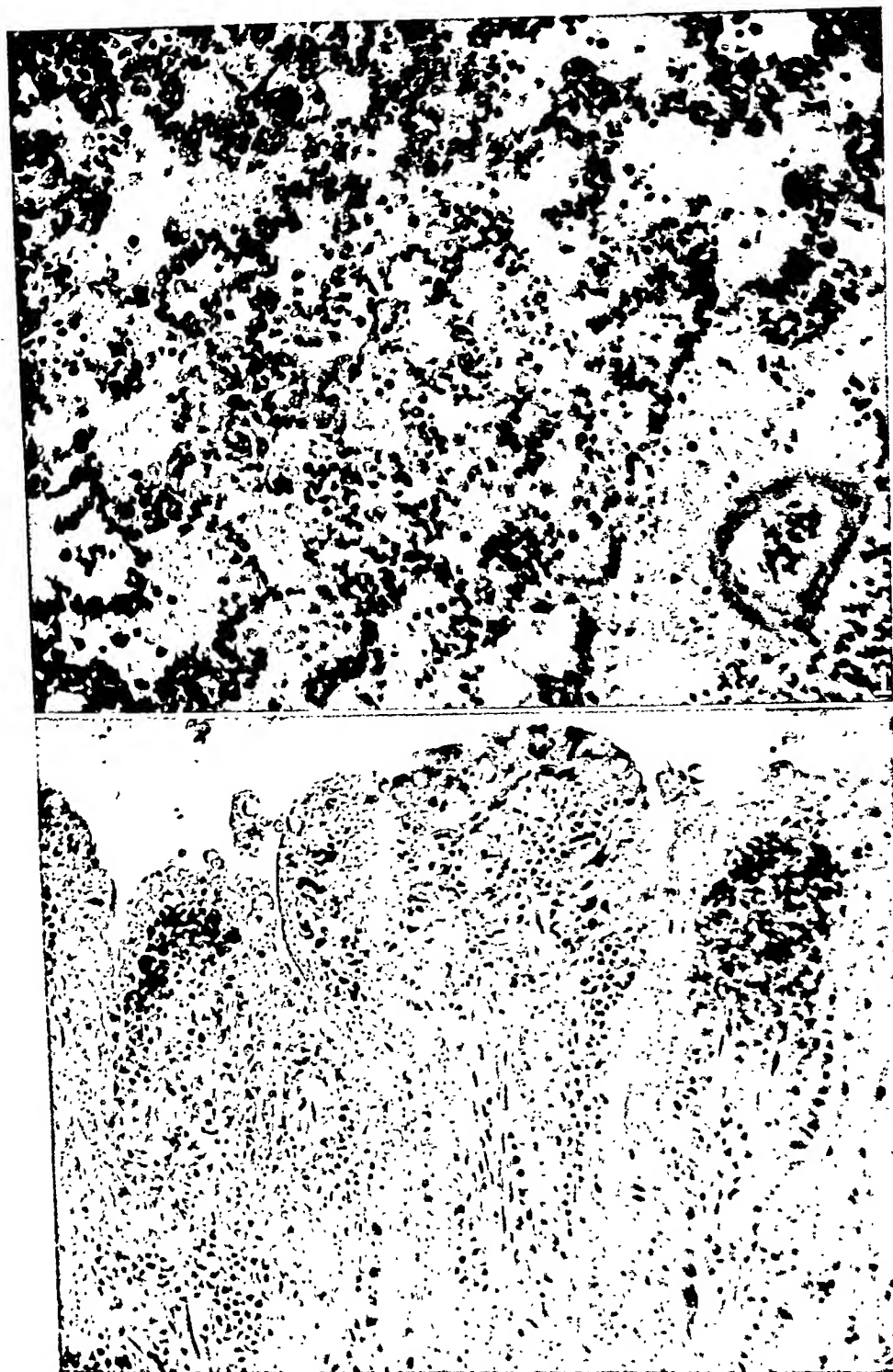
FIG. 1. Section through lung of ferret infected intranasally with Rift Valley fever virus. The organ appeared consolidated in the gross. There are thickening and hyperemia of the alveolar walls. The alveoli contain edema fluid and a scanty exudate of large mononuclear cells. Edema of the arterial wall is pronounced. (Eosin-methylene blue. M plate, B filter.  $\times 210$ .)

FIG. 2. Section of the mucosa of the small intestine of an infected ferret in which hemorrhagic enteritis was present at autopsy. There is hyperemia of the vessels and extravasation of erythrocytes into the villi. (Eosin-methylene blue. M plate, 2 G filters.  $\times 210$ .)

##### PLATE 17

FIG. 3. Section of liver of an infected ferret showing mid-zonal hemorrhagic necrosis. (Eosin-methylene blue. M plate, 2 G filters.  $\times 110$ .)

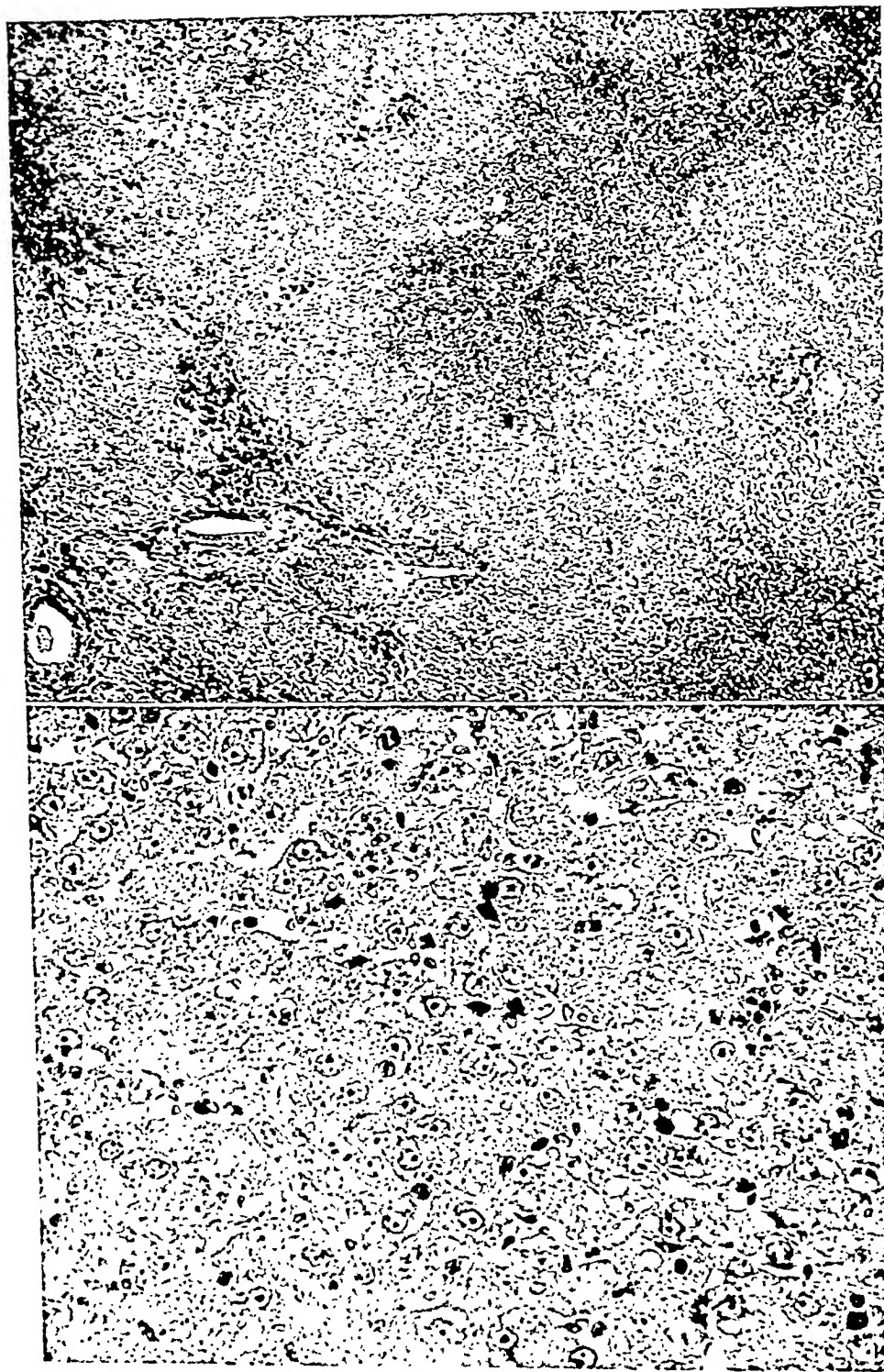
FIG. 4. Section of the liver of an infected ferret in which a rather uniform cytoplasmic lesion is present. The cells are pale, granular, and contain many vacuoles. (Eosin-methylene blue. M plate, 2 G filters.  $\times 460$ .)



Photograph by Louis Schmidt

(Francis and Mazell. Rift Valley fever)





Photographed by Louis Schmitt

(Francis and MacCall's Rift Valley fever)



## INTERSTITIAL BRONCHOPNEUMONIA

### II. PRODUCTION OF INTERSTITIAL MONONUCLEAR PNEUMONIA BY THE BORDET-GENGOU BACILLUS\*

By DOUGLAS H. SPRUNT, M.D., DONALD S. MARTIN, M.D., AND  
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of Medicine, Durham)

PLATE 18

(Received for publication, June 24, 1935)

The rôle of the Bordet-Gengou bacillus as the primary etiologic agent of pertussis has been questioned by McCordock (1) and Rich (2). The latter worker reviews the literature on the subject and draws attention to the fact that the criterion of the successful production of pertussis in animals has often been the presence of a paroxysmal cough or "whoop." This "whoop," he believes, can be caused by other types of bacteria if sufficient numbers are present in the trachea and bronchi. This idea is supported by the work of Blake and Cecil (3) who state that monkeys injected intratracheally with Pfeiffer's bacillus have at times a severe racking cough. Furthermore Brown (4) describes an instance of a child infected with *Bacillus bronchisepticus* who had typical symptoms of pertussis.

Certain similarities between pertussis and measles or other virus diseases led McCordock (1) and Rich (2) to suggest that pertussis may be a virus infection. Rich also suggests the permanent immunity following recovery, the extreme infectivity of the causative agent of pertussis, and the occurrence of encephalitis as an occasional complication are evidences in favor of the idea that whooping cough is caused by a virus. Both investigators cite as additional evidence of the virus nature of the causative agent of the disease the presence of intranuclear inclusions and interstitial mononuclear pneumonia in the lungs of children dying of pertussis.

In spite of the observations mentioned above direct evidence that a virus is the primary etiologic agent of pertussis is still lacking. More recently Rich, Long, and their associates (5) failed to produce symptoms suggestive of whooping cough in chimpanzees by inoculation of filtered nasal washings from a case of

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pertussis, but were successful in infecting chimpanzees by means of unfiltered nasal washings and pure cultures of the Bordet-Gengou bacillus. MacDonald and MacDonald (6) also failed to produce pertussis in children with filtrates but succeeded with pure cultures of the bacillus. Culotta and his associates (7) succeeded in infecting monkeys with the organism, but neither filtered nor unfiltered nasal washings of children with pertussis proved to be infectious.

In a previous paper (8) we showed that certain bacterial toxins are capable of producing an interstitial mononuclear pneumonia similar to that caused by the viruses. It was pointed out in that communication that the toxins which caused this type of pneumonia had in common with the viruses certain properties; *e.g.*, the ability to produce a prolonged or a permanent immunity. This led us to the study of the lesions which could be produced in the lungs of rabbits by the Bordet-Gengou bacilli and the typhoid bacilli, both of which organisms, like viruses and toxins, possess strong antigenic properties.

The work described in this paper was designed to show that the Bordet-Gengou bacillus of itself can produce an interstitial mononuclear pneumonia and that the occurrence of this type of reaction in the lungs cannot be used to support the idea that pertussis is a virus disease.

### *Methods and Materials*

The animals used in the experiments were young adult rabbits which had been tested for the presence or absence of *Bacterium leptosepticum* and *Bacillus bronchisepticus* by the method described in the first paper of this series. All rabbits harboring these organisms were excluded.

*Medium.*—The medium for the cultivation of the pertussis bacillus was similar to that described by Bordet and Gengou (9) with the exception that 25 per cent defibrinated human blood was substituted for the horse blood.

*Organisms.*—The strain of Bordet-Gengou bacillus used in this work was obtained from Dr. W. A. Jameson of the Eli Lilly Co. and the virulence of the organism was maintained by growing it on the above described medium. A virulent strain of *Bacillus typhosus* which had been kept on beef infusion agar slants since its isolation in 1918 was employed.

*Lysates and Vaccines.*—Various methods were used in preparing toxic extracts from the Bordet-Gengou bacillus. The bacteria obtained from the 72 hour growth on 30 Bordet-Gengou slants were scraped off into 10 cc. of sterile distilled water. The suspension was then frozen and thawed 6 times. No disintegration of the bacilli was found in a stained smear. The supernatant fluid and the sediment, both of which were sterile, were kept for animal inoculation. A second lysate

was made by the method described by Teissier and his associates (10). The organisms were powdered by grinding with sterile salt in a mortar. The powder was then suspended in enough distilled water to make the suspension isotonic after which it was kept at room temperature for 24 hours. The supernatant fluid was sterile and clear. This fluid was kept for animal inoculation. Commercial vaccines prepared by Eli Lilly and Co. according to the Krueger and the Sauer techniques were employed.

*Dose.*—In every instance the total volume of the material injected into each animal was 1 cc. The pertussis organism was grown for 48 hours on 1 or 2 Bordet-Gengou agar slants and then suspended in sterile normal saline. The number of organisms obtained in this manner varied between 50 and 100 billion. The typhoid bacilli were grown on beef infusion agar slants. The 18 hour surface growths of 2 slants suspended in 10 cc. of saline were used. After several passages through the rabbits' lungs, an extensive lesion could consistently be obtained by use of the growth from  $\frac{1}{4}$  of an agar slant.

*Inoculation.*—The inoculations were made intratracheally as described in the first paper of this series.

*Necropsy.*—The necropsies were done as described in the preceding paper except that, in addition to the routine cultures of the lungs, the bronchi and lungs were tested for the presence of the Bordet-Gengou bacillus by means of culture on Bordet-Gengou plates.

*Fixation and Stains.*—After the lungs were removed from the body they were inflated with air and fixed in Zenker's solution (Helly's modification). After fixation the lungs were embedded in paraffin. Sections were stained with hematoxylin and eosin, Mallory's eosin-methylene blue, MacCallum's bacterial stain, and by the method described by Brown and Brenn for staining Gram-negative bacilli.

#### EXPERIMENTAL

The experiments were designed to study the effect of living Bordet-Gengou organisms and lysates and vaccines of these bacteria on the lungs of rabbits. Similar experiments were performed with *Bacillus typhosus*. The latter organism was chosen because typhoid may be followed by an encephalitis and permanent immunity similar to those following pertussis. Although we were not able to find reports of good morbid anatomical studies of the encephalitis following typhoid fever, the clinical histories reported by Wieland (11), Hillemand and his associates (12), and others indicate that this encephalitis is similar to that following pertussis.

*Experiment 1.*—22 rabbits were used in the experiment and each received the organisms from 1 or 2 Bordet-Gengou agar slants suspended in 1 cc. of saline.

The animals were killed at intervals of from 12 hours to 2 weeks after inoculation. The majority, however, were killed at 72 and 96 hours.

*Morbid Anatomy. Gross.*—The lungs from the rabbits killed at the end of 12 and 24 hours show little except some congestion and a few areas of hemorrhage. After 72 hours there is usually an area of consolidation which as a rule is near the hilus of the left lung posteriorly but which at times may involve other portions of the lungs. In some animals such areas are firm and dark purple, in others necrotic.

*Microscopic.*—The lungs removed 12 hours after death show a number of polymorphonuclear cells. These are most prevalent around the bronchi but are also in the alveoli, the interstitial tissue, and the perivascular lymphatics. A number of red blood cells and an occasional mononuclear cell are present in a few focal areas.

At the end of 3, 4, and 5 days the picture is essentially similar to that produced with bacterial toxins (8). The animals receiving a large dose as a rule show necrosis of lung tissue while those receiving a small dose show evidence of proliferation. The proliferative lesion will be described first. Some of the alveoli are lined with cells having vesicular nuclei, small nucleoli, and scanty, poorly stained cytoplasm. Some of these cells are undergoing mitosis but mitotic figures are not as numerous as in the case of tissues injured by bacterial toxins. The lumina of the alveoli, both those with proliferating lining cells and those without, are filled with mononuclear cells (Fig. 1). Such cells are similar to those attached to the walls except that their cytoplasm is more plentiful. They resemble monocytes and macrophages. Intermixed with these cells are polymorphonuclear cells in various stages of degeneration. In some instances there is rather extensive hemorrhage. All of the perivascular lymphatics are filled with lymphocytes and in many instances the walls of the blood vessels are infiltrated with them. In no instance, however, is a thrombus found. There is a definite increase in lymphocytes around the bronchi and bronchioles. The mucosa of the bronchi and bronchioles is infiltrated with polymorphonuclear cells, and occasionally there is a hyperplasia of the lining epithelium.

The animals which received the large dose show infarct-like areas of necrosis. This necrosis, however, is not due to thrombosis. Although more extensive involvement of the blood vessels is seen than described above, no thrombi are observed. That the necrosis followed the proliferation is evidenced by the fact that the alveolar walls are thickened even in the necrotic areas. The areas of necrosis are infiltrated in some instances with polymorphonuclear cells, but this is less extensive than in the toxin experiment previously reported (8). In other portions of the lungs rather extensive hemorrhage is seen. After 1 and 2 weeks the picture is more or less similar to that already described except that the proliferation had become more marked. At first sight the number of bronchioles seem to be increased throughout the lung. On closer inspection these are seen to be alveoli lined with cells which might be mistaken for cells lining the bronchioles (Figs. 2 and 3). There are still some polymorphonuclear cells present, but they are not as numerous as in the earlier lesions. The perivascular lym-

phatics are still filled with lymphocytes. The proliferation of the cells lining the alveoli has in some instances proceeded to such an extent that they form a syncytium of cells. There are a few multinuclear or giant cells similar to those found in the toxin experiment. The lymphoid tissue around the bronchi is still increased. Only a slight amount of fibrosis is found and it is thought that the structure of the lungs would return to normal if sufficient time were allowed.

In none of the rabbits' lungs were inclusion bodies found. The Bordet-Gengou bacilli were only recovered in animals killed within 48 hours after inoculation. No other organisms were found in the lung by means of stains or cultures.

*Experiment 2.*—18 rabbits were inoculated intratracheally with the various lysates and vaccines. The animals were killed 72 and 96 hours after inoculation. Microscopically a few areas of mononuclear cells are found.

*Morbid Anatomy.*—8 rabbits were injected intratracheally with a suspension of the typhoid bacilli. The first rabbit was inoculated with the material from 2 agar slants. A small lesion resulted and a large number of typhoid bacilli were cultured from it. After passage of the bacilli through several animals the inoculum had to be reduced from 2 agar slants to  $\frac{1}{4}$  of one agar slant as a larger dose caused death of the animals in less than 24 hours. The animals which received bacilli from  $\frac{1}{4}$  agar slant had lesions about the same size as those which received the pertussis organisms. Typhoid bacilli did not grow out on solid media smeared with the lung tissue. However, in some instances the bacilli could be recovered from broth tubes inoculated with a large piece of lung.

*Morbid Anatomy.*—The lungs of the rabbits inoculated with typhoid bacilli resemble in every respect those infected with pertussis bacilli described in Experiment 1.

*Controls.*—In order to be certain that the reactions described in the lungs were due to the organisms injected and not to a latent virus or to extraneous materials which may have been washed from the slants, the following control experiments were conducted. In all instances the animals were killed 72 hours after inoculation.

*Medium.*—2 rabbits were inoculated with material prepared in the following manner. 2 cc. of sterile normal saline were added to an uninoculated Bordet-Gengou slant. The slant was scraped with the inoculating loop so that a blood-tinged cloudy suspension was obtained. No lesions were produced by intratracheal inoculation of this material.

*Avirulent Organisms.*—6 rabbits were inoculated with avirulent Bordet-Gengou organisms. No lesions with the exception of a few small hemorrhages were found in the lungs of these rabbits.

*Virus Control.*—A rabbit's lung with a typical lesion was triturated with an abrasive. A 10 per cent suspension was made, and 1 cc. of this was injected into

each of 3 rabbits. The lungs of these rabbits show nothing more than small hemorrhagic areas.

#### DISCUSSION

The morbid anatomical changes described at this time are similar to those previously described as resulting from the action of bacterial toxins (8). Through the courtesy of Drs. Rivers and Francis we had the opportunity of comparing our sections with theirs showing lesions produced by the viruses of psittacosis and epidemic influenza. The lesions are similar. A study of the lung lesions found in several cases of pertussis as well as descriptions of the pneumonia in pertussis by McCordock (1) and Rich (2) led us to believe that the lesions in the lungs of rabbits inoculated with pertussis bacilli could not be differentiated from those found in human beings with pertussis. On the basis of these observations we feel justified in maintaining that the presence of an interstitial mononuclear pneumonia in pertussis is not evidence that pertussis is a virus disease.

The similarity of the pneumonia caused by pertussis bacilli to that produced by bacterial toxins led us to try to determine whether a toxin capable of producing such a lesion could be obtained from the Bordet-Gengou bacillus. We have not yet been able to derive a toxin from the organism *in vitro* and our experiments with the lysates and vaccines are not at all conclusive in this regard. Nevertheless the inability to recover viable Bordet-Gengou bacilli 48 hours after inoculation, the ease with which typhoid bacilli were recovered from the lungs of rabbits which had slight lesions, and the difficulty in recovering them from good lesions suggest that the reactions are due to some substances liberated by the death of the organism in the animal body. It is interesting to note that Bordet and Gengou (13) also failed to recover the pertussis organism from the peritoneum of guinea pigs inoculated with a fatal dose.

The possibility that the lysates and the vaccines retain their immunizing properties in spite of losing their toxic action is being investigated. The fact that an interstitial mononuclear pneumonia can be produced in rabbits with pure cultures of the Bordet-Gengou bacillus makes possible such an investigation.

Because of certain similarities between the Bordet-Gengou bacillus,

*Bacillus bronchisepticus* and *Bacterium leprosepticum*, and since many rabbits harbor the last two organisms mentioned, the results of much of the previous work on pertussis in animals have been questioned. The presence of these organisms in our experiments was carefully avoided, inasmuch as the rabbits' nares were cultured before the experiments were started, and the lungs at necropsy were cultured and stained in order to demonstrate their absence. Whenever these organisms were encountered the rabbits were discarded, and we believe that errors from this source have been obviated.

In experimental work of this nature one must consider the possibility of the stimulation by the experimental procedures of a latent virus in the animal body. In our work, however, the excitation of a latent virus can be excluded by the absence of inclusion bodies in the lungs and by the inability of the material taken from typical lesions to incite similar lesions in other rabbits.

McCordock (1) and Rich (2) questioned the relation of the inclusions in the lungs to the etiologic agent of pertussis. The failure to find inclusions in our experimental animals as well as the fact that Von Glahn and Pappenheimer (14) have described similar inclusions in the lungs of an adult not affected with pertussis lead us to believe that the intranuclear inclusions found in patients dying of pertussis bear no etiologic relation to the disease.

The more or less permanent immunity induced by an attack of pertussis and the encephalitis that occasionally follows the disease are cited by Rich (2) as reasons for believing that pertussis is a virus infection. We have already indicated that both of these phenomena are associated with typhoid fever. Hence it is not unreasonable to suppose that a bacterium instead of a virus may be the cause of whooping cough.

#### CONCLUSIONS

Pure cultures of Bordet-Gengou bacilli produce in rabbits an interstitial mononuclear pneumonia which cannot be differentiated from that occurring in children dying of pertussis or from that caused in animals by the viruses of epidemic influenza and psittacosis. A similar pneumonia can be produced in rabbits by typhoid bacilli.



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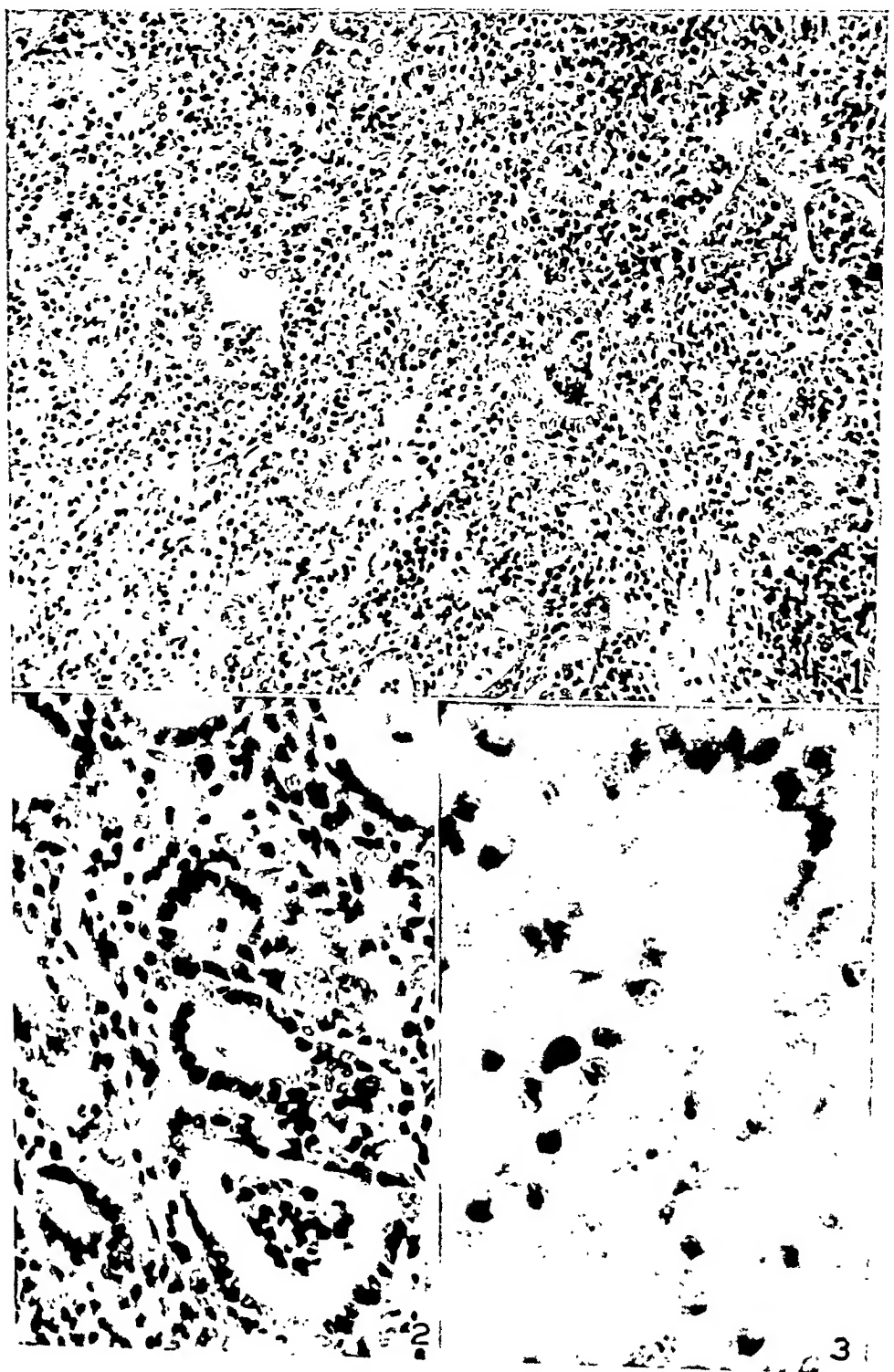
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## EXPLANATION OF PLATE 18

FIG. 1. Section of rabbit's lung 96 hours after intratracheal inoculation of Bordet-Gengou bacilli. There is a marked increase of the interstitial cells and a proliferation of the cells lining the alveoli.  $\times 210$ .

FIG. 2. Section of rabbit's lung, killed 7 days after intratracheal inoculation of Bordet-Gengou bacilli, showing alveoli lined with proliferating cells so that the alveoli resemble a bronchiole. There is also an increase in the interstitial tissue.  $\times 450$ .

FIG. 3. Section of rabbit's lung, killed 7 days after inoculation of Bordet-Gengou bacilli. Note the mononuclear cells and also the proliferation of the lining cells of the alveolus.  $\times 830$ .



(Sjunt et al.: Interstitial lymphoma, p. 11)



# HEMOGLOBIN REGENERATION IN THE CHRONIC HEMORRHAGIC ANEMIA OF DOGS (WHIPPLE)

## I. THE EFFECT OF IRON AND PROTEIN FEEDING

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(Received for publication, June 3, 1935)

The evaluation of the potency of a large variety of materials for hemoglobin regeneration in the chronic hemorrhagic anemia of dogs by Whipple and his associates (1) has stimulated further investigation of this condition. The less startling effect of liver feeding in the various types of human secondary anemia (2) raises an interesting question concerning the metabolism of iron and hemoglobin. Why, for example, is liver more effective in these anemic dogs than inorganic iron?

An analysis of the daily intake of calories, protein, iron and copper in the diets of 60 representative protocols taken from the publications of the Rochester laboratory, shows for the fruits, vegetables and dairy products, a close correlation between hemoglobin regeneration and the amount of iron ingested when the factor of the availability of these forms of food iron stressed by Elvehjem and his associates (3) is considered. With the glandular, however, and, to a less extent, the muscular foods other factors besides their iron content and its availability to the animal appear to be operating. Perhaps the chronic hemorrhage, in the removal of large amounts of blood constituents other than hemoglobin, and the unfavorable diet used in developing a slowly and constantly regenerating anemia, lead to a deficiency of materials besides iron involved directly or indirectly with hemoglobin formation. In such multiple deficiencies the addition of one of the materials which is lacking causes improvement in the animal's condition. This phenomenon may be considered as a type of mass action effect illustrated by the relationship of copper

and iron in the milk anemia of young rats (4) or the influence of the relative amounts of calcium, phosphorus and iron present in the diet on the absorption of these materials (5) by the gastro-intestinal tract.

The standard basal salmon bread ration supplies a daily copper intake of 1.0 to 1.5 mg. A copper deficiency in these dogs is unlikely considering the small amounts of this element required for iron utilization by the anemic rat. Since liver is more effective than iron in these anemic dogs and the two together develop a summation response (6) our attention is turned to some other, possibly organic, factor present in the liver which may be effective in hemoglobin regeneration. The important observation (7) that the iron-poor liver of the horse made anemic by bleeding, loses none of its potency for hemoglobin regeneration in anemic dogs, stimulates this search for another factor in a multiple deficiency state. The rôle of a protein, namely casein, was studied as the first possibility.

### *Methods*

A detailed description of the care of the dogs, the production and maintenance of the anemia and the experimental procedure is unnecessary since Whipple's methods have been duplicated to the best of our ability (8, 9). Briefly, dogs were fed the salmon bread ration developed in the Rochester laboratory and were bled from the femoral artery at the intervals and in the amounts necessary to produce, at the end of 8 to 12 weeks' time, an anemia with a hemoglobin level about one-third of the normal and a rate of regeneration not greater than 2 gm. of hemoglobin a week. Diets to be assayed were fed daily for 2 weeks. The hemoglobin producing power was measured by the amount of hemoglobin bled in order to restore the blood hemoglobin to its original level and to return the rate of regeneration to the basal of 2 gm. per week. After a special diet is discontinued, bleeding during a period of 2 or 3 weeks is usually required while the animal is eating the basal ration.

Hemoglobin was estimated with a Sahli-Leitz hemoglobinometer calibrated so that 14 gm. of hemoglobin equalled 100 per cent. These readings were checked with the blood iron method described by Hanzal (10). Red blood cell and white blood cell counts were performed with Bureau of Standards apparatus. Hematocrit and plasma volume determinations employed the methods described by Hooper, Smith, Belt and Whipple (11) using the brilliant vital red dye and 15 cc. graduated centrifuge tubes containing 2 cc. of 1.6 per cent sodium oxalate. Blood films were made on cover slips previously coated with brilliant cresyl blue. Blood for these determinations was obtained from the femoral instead of the jugular veins. All of these observations were made on each dog on a regular day each week. The daily food consumption was recorded.

The care necessary to maintain such anemic dogs in a constant condition for assaying the hemoglobin producing power of a material requires emphasis. Whipple has called attention to the effect of a preceding favorable diet on a subsequent hemoglobin response to another diet fed after an interval of many weeks. If other factors in the animal's condition besides the hemoglobin level such as the red blood cell count, the reticulocyte level, the white blood cell count, hematocrit, plasma volume, body weight, general appearance and activity, are considered carefully, this source of error can be eliminated.

The basal diet was restricted to the salmon bread developed by Whipple and Robscheit-Robbins (9) with the exception of Dog 4 which received in addition 430 gm. of fresh, whole, pasteurized milk daily. The daily diet was computed on the basis of 80 calories per kilo of body weight. Distilled water was kept in the cages at all times and was used in the preparation of the salmon bread. The cages were small enough to limit activity, and the dog room was well ventilated. An anthelmintic dose of tetrachlorethylene was administered routinely at 6 month intervals.

All of the diets were analyzed for their content of iron, copper and protein nitrogen. Iron was determined by the method employed by Elvehjem (12), the accuracy of which has been proved in a comparative study of several analytical procedures (13). The Biazzo method for copper analysis described by Elvehjem and Lindow (14) was used. Protein nitrogen was determined by macro Kjeldahl determinations of the difference between the total nitrogen and the nitrogen in a trichloroacetic acid extract of the material. Caloric values were not determined; food table values were used (15). The foods employed had the following nutritional properties based on the weight as fed: salmon bread, 4 calories per gm., 9 per cent protein, 3 mg. per cent iron, 0.4 mg. per cent copper; milk, 0.7 calories per gm., 3.3 per cent protein, 0.05 mg. per cent iron, 0.015 mg. per cent copper; casein (Lister), 3.4 calories per gm., 84.5 per cent protein, 4.3 mg. per cent iron, 0.4 mg. per cent copper; frozen beef liver, 1.3 calories per gm., 22.5 per cent protein, 5 mg. per cent iron, 2.6 mg. per cent copper. Iron was added to the dog's ration in the form of a solution of ferric ammonium citrate in dilute hydrochloric acid with an iron concentration of 10 mg. per cc.

With these constituents, additions to the basal ration were planned whereby diets of liver or of casein and inorganic iron were obtained in which the animal received the same amounts of iron and protein. The hemoglobin response to these diets which quantitatively contained equal amounts of iron and protein of different sources, was compared with the response to the addition of an equal amount of inorganic iron without any additional protein. Calculations based on surface area instead of body weight have yielded no further information.

The weekly basal rate of hemoglobin production on the salmon bread diet was determined for each dog during 8 week periods and was found to be as follows: Dog 1, 1.8 gm.; Dog 2, 2.5 gm.; Dog 3, 1.7 gm.; Dog 4, 2.2 gm. This gave an average of 2 gm. a week arising from the salmon bread ration. In order to compare the effect of additions to the basal ration, this control rate of regeneration has been subtracted from the total hemoglobin removed by bleeding during the

experimental feeding periods. The analysis of Whipple's protocols mentioned above shows that hemoglobin production is correlated with the additional iron but not with the total iron of the diet period.

### RESULTS

Chart 1 shows the results obtained with two of the four dogs studied. The addition of milk to the basal diet of Dog 4 was originally planned

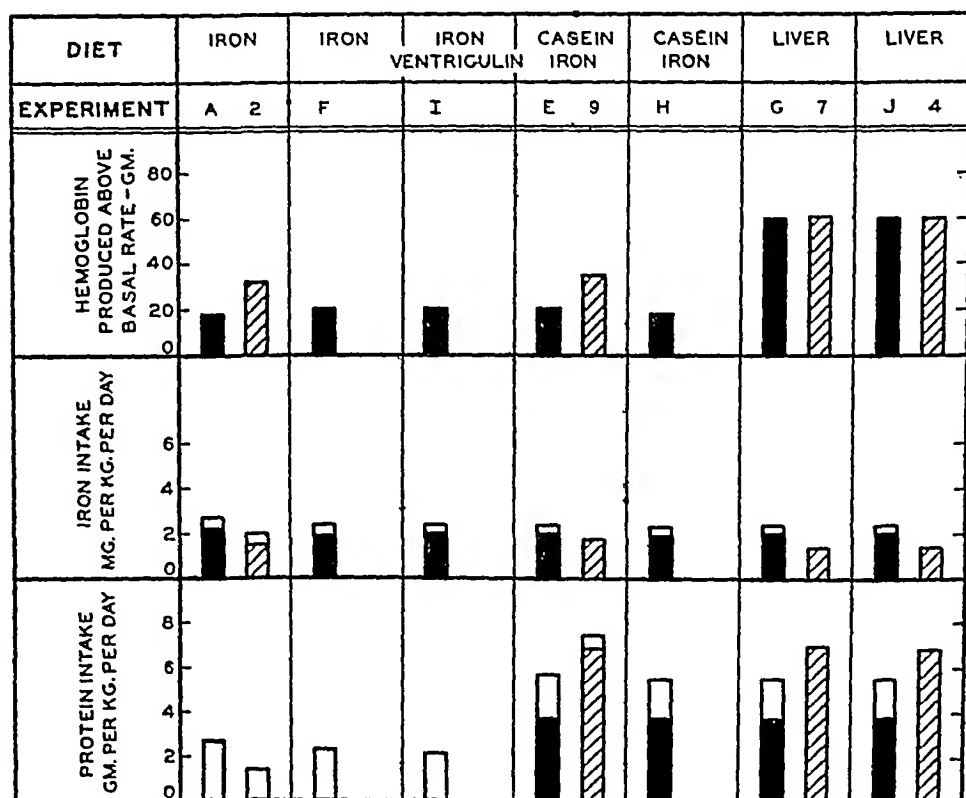


CHART 1. Hemoglobin produced above the basal rate in response to the addition to the diet of amounts of iron, casein-iron and liver during 2 week periods, representing equivalent quantities of iron and protein nitrogen. The open areas represent the iron or protein intake arising from the salmon bread and milk component of the diet. The numbered and cross-hatched experiments are on Dog 1, the lettered and full block ones on Dog 4.

as a check on the possibility of a protein (casein) deficiency in the salmon bread which contains only 9 per cent protein of fish and cereal origin. The results were, however, the same for all of these dogs. Seven experiments on Dog 4 and four experiments on Dog 1 are shown in the chart. Dogs 2 and 3 showed similar responses. Dog 4 also

TABLE I

*The Response of Dog 4 to the Addition of Equivalent Amounts of Iron and Protein, in the Form of Ferric Chloride, Casein and Liver, to the Basal Milk and Salmon Bread Diet*

| Periods of 1 week<br>Diet per day in gm. |      |            | Weight<br>kg. | Plasma volume<br>cc. | R.B.C.<br>mil-<br>lion | R.B.C. hematocrit<br>per cent |    | Hemoglobin        |             |                                     | Food intake per kg. per day |              |              |              |          |               |
|--|------|------------|---------------|----------------------|------------------------|-------------------------------|----|-------------------|-------------|-------------------------------------|-----------------------------|--------------|--------------|--------------|----------|---------------|
|  |      |            |               |                      |                        |                               |    | Level<br>per cent | Bled<br>gm. | Produced above<br>basal rate<br>gm. | Iron                        |              | Protein      |              | Calories | Copper<br>mg. |
|  |      |            |               |                      |                        |                               |    |                   |             |                                     | Total<br>mg.                | Added<br>mg. | Total<br>gm. | Added<br>gm. |          |               |
|  |      |            |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| Experiment F                             |      |            |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| Bread S                                  | 215  | Milk 430   | 15.3          | —                    | 7.3                    | 26.2                          | 58 | 2.0               |             |                                     | 0.43                        |              | 2.2          |              | 75       | 0.06          |
| "  | 250  | " 430      | 15.7          | 926                  | 6.6                    | 30.2                          | 55 | 1.2               |             |                                     | 0.49                        |              | 2.3          |              | 82       | 0.07          |
| "  | 250  | " 430      | 15.4          | —                    | 7.3                    | 33.3                          | 61 | 1.9               |             |                                     | 2.4                         | 1.9          | 2.4          | 0            | 84       | 0.07          |
| Iron                                     | 0.03 |            |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| "  | 250  | Milk 430   | 15.6          | —                    | 7.5                    | 34.8                          | 67 | 0.8               |             |                                     | 2.4                         | 1.9          | 2.3          | 0            | 83       | 0.07          |
| Iron                                     | 0.03 |            |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| "  | 250  | Milk 430   | 15.6          | —                    | 6.0                    | 28.0                          | 55 | 23.5              |             |                                     | 0.5                         |              | 2.3          |              | 83       | 0.07          |
| "  | 250  | " 430      | 15.8          | 908                  | 5.0                    | 25.7                          | 50 | 0.8               |             |                                     | 0.49                        |              | 2.3          |              | 82       | 0.07          |
|  |      |            |               |                      |                        |                               |    |                   |             | 19                                  |                             |              |              |              |          |               |
| Experiment E                             |      |            |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| Bread S                                  | 250  | Milk 430   | 15.1          | 837                  | 5.5                    | 23.2                          | 46 | 16.9              |             |                                     | 0.51                        |              | 2.4          |              | 86       | 0.07          |
| "  | 250  | " 430      | 15.1          | —                    | 4.9                    | 21.7                          | 44 | 1.6               |             |                                     | 0.51                        |              | 2.4          |              | 83       | 0.07          |
| "  | 200  | " 430      | 15.6          | —                    | 5.3                    | 24.3                          | 49 | 1.5               |             |                                     | 2.42                        | 2.0          | 5.9          | 3.8          | 74       | 0.07          |
| Casein                                   | 70   | Iron 0.03  |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| Bread                                    | 250  | Milk 430   | 15.9          | 821                  | 5.3                    | 28.6                          | 58 | 0.7               |             |                                     | 2.42                        | 2.0          | 5.7          | 3.7          | 80       | 0.07          |
| Casein                                   | 70   | Iron 0.03  |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| Bread                                    | 250  | Milk 430   | 15.9          | —                    | 5.7                    | 26.5                          | 50 | 16.3              |             |                                     | 0.48                        |              | 2.3          |              | 81       | 0.07          |
| "  | 250  | " 430      | 15.6          | —                    | 5.2                    | 26.9                          | 50 | 0.7               |             |                                     | 0.49                        |              | 2.3          |              | 83       | 0.07          |
| "  | 250  | " 430      | 15.7          | 870                  | 4.6                    | 23.7                          | 49 | 10.8              |             |                                     | 0.50                        |              | 2.3          |              | 83       | 0.07          |
|  |      |            |               |                      |                        |                               |    |                   |             | 20                                  |                             |              |              |              |          |               |
| Experiment G                             |      |            |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| Bread S                                  | 250  | Milk 430   | 15.8          | 917                  | 5.75                   | 30.8                          | 60 | 1.7               |             |                                     | 0.48                        |              | 2.3          |              | 82       | 0.06          |
| "  | 200  | " 430      | 16.7          | —                    | 7.65                   | 32.9                          | 78 | 1.7               |             |                                     | 2.37                        | 2.0          | 5.3          | 3.4          | 85       | 0.44          |
| Liver                                    | 250  | Iron 0.021 |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| Bread                                    | 200  | Milk 430   | 16.9          | —                    | 6.05                   | 31.9                          | 72 | 31.7              |             |                                     | 2.49                        | 2.1          | 5.9          | 4.0          | 88       | 0.51          |
| Liver                                    | 300  | Iron 0.021 |               |                      |                        |                               |    |                   |             |                                     |                             |              |              |              |          |               |
| Bread                                    | 250  | Milk 430   | 16.8          | —                    | 5.55                   | 31.1                          | 66 | 18.4              |             |                                     | 0.46                        |              | 2.2          |              | 77       | 0.06          |
| "  | 250  | " 430      | 16.5          | —                    | 5.55                   | 31.8                          | 65 | 1.4               |             |                                     | 0.47                        |              | 2.2          |              | 78       | 0.06          |
| "  | 250  | " 430      | 16.6          | 862                  | 6.25                   | 33.2                          | 67 | 3.8               |             |                                     | 0.46                        |              | 2.2          |              | 78       | 0.06          |
| "  | 250  | " 430      | 16.5          | 785                  | 5.3                    | 27.6                          | 56 | 15.4              |             |                                     | 0.47                        |              | 2.2          |              | 78       | 0.06          |
| "  | 250  | " 430      | 16.1          | 898                  | 6.25                   | 31.7                          | 63 | 1.6               |             |                                     | 0.48                        |              | 2.3          |              | 80       | 0.06          |
| "  | 250  | " 430      | 16.9          | 871                  | 5.9                    | 30.5                          | 60 | 1.8               |             |                                     | 0.45                        |              | 2.2          |              | 77       | 0.06          |
|  |      |            |               |                      |                        |                               |    |                   |             | 60                                  |                             |              |              |              |          |               |



differs from the other dogs in the use of inorganic iron with the liver diets (necessary to obtain an iron and protein intake equivalent to the casein-iron periods). Table I gives in detail the weekly observations and the diet for three representative experiments on Dog 4. In these three feeding periods of iron, casein-iron and liver, the iron addition is constant at about 2 mg. per kilo per day and the protein addition in the last two experiments is constant at about 3.7 gm. per kilo per day. The excess hemoglobin produced above the basal level is for the inorganic iron experiment 19 gm., for the inorganic iron and casein period 20 gm., and for the addition of liver to the basal ration 60 gm. It is obvious that casein does not contain the important factor present in liver, since its addition to the diet does not improve on the response to iron alone. These studies confirm the reports from Whipple's laboratory of the greater effectiveness of liver than of inorganic iron for hemoglobin production in this chronic hemorrhagic anemia of dogs. Furthermore, in liver and iron feeding periods representing the same quantitative iron intake, the liver is more effective. Hence, the difference between liver and inorganic iron cannot be due to a larger dose or a better availability of iron in the liver periods. It is well to point out that the responses recorded are submaximal to the substance in question. Experiments in feeding larger amounts of each of these materials have yielded more hemoglobin than is recorded for this series in Chart 1. Such an assay of necessity must employ a submaximal response.

Chart 1 illustrates the differences among dogs in their powers of hemoglobin production which Whipple often mentions. Although Dog 1 received less iron in all experiments than Dog 4, its hemoglobin responses are more marked in the inorganic iron periods. The constant quantitative nature of this hemoglobin response is shown by Dog 4 (solid blocks in the chart) with three iron, two casein-iron and two liver experiments. Such an animal has, therefore, a hemorrhagic type of anemia with the accuracy and sensitivity necessary for assay purposes comparable to the nutritional anemia type obtained in the young rat by an exclusive milk diet (16).

#### DISCUSSION

As a further confirmation of the rôle of protein in the form of casein in hemoglobin production in this hemorrhagic anemia, two

dogs, raised in this laboratory, were studied in a similar manner to the other four except that Cowgill's (17) dog diet was used in the place of salmon bread. Since only two dogs have been observed in this experiment, these observations will be reported only briefly. This Cowgill ration has the following composition: casein (Lister) 37.2 per cent, sugar 34.3 per cent, lard 21.7 per cent, cod liver oil 2 per cent, agar-agar 2.4 per cent, salt mixture (McCollum-Simmonds No. 185, without ferric citrate, the same salt mixture used in the salmon bread) 2.4 per cent. The nutritional properties of this diet are 4.7 calories per gm., 30.2 per cent protein, 2 mg. per cent iron and 0.13 mg. per cent copper. Two yeast vitamin tablets (Harris) were added to the daily ration (represents 0.03 mg. of iron). This diet was fed in amounts allowing the 80 calories per kilo of body weight as specified by Cowgill. This is a high protein diet which allows about 5 gm. of casein per kilo daily as compared to the 2 gm. of fish and cereal protein afforded by the salmon bread diet. Protein deficiency is less likely, therefore, to be a factor when this diet is fed. After exhaustion of the hemoglobin reserves by bleeding, these dogs showed the same constant basal hemoglobin regeneration rate of 2 gm. a week as shown by the dogs eating the salmon bread ration. Mayerson and Laurens (18) reported that anemic dogs would neither maintain their hemoglobin level nor survive long on the Cowgill ration. It should be noted, however, that a different salt mixture was used in their diet. Some anemic dogs have refused to eat the Cowgill diet in this laboratory, but all of the six dogs mentioned in this report ate this ration well. They maintained their weight on this diet and showed, as mentioned, the same constant basal hemoglobin regeneration rate. The animals which had previously been stabilized on the salmon bread diet, showed an excess hemoglobin production of about 5 gm. a week for a 2 week feeding period with the Cowgill diet. Since its iron content is lower than in the salmon bread diet, this last observation again suggests a deficiency in the latter ration. The availability of the iron contained in the two diets does not explain this difference, since the bipyridyl reaction (3) shows about 50 per cent availability for the iron of both diets.

If protein of the amino acid content of casein were one of the important factors, animals whose anemia was stabilized on the Cowgill ration might be expected to show a response to the addition of in-

organic iron approximating more closely their response to liver than in the case of dogs living on the salmon bread diet. Accordingly fresh frozen beef liver (275 gm. daily) was substituted during 2 weeks for the casein in the Cowgill ration which kept the daily protein intake constant and at the same time allowed an additional iron intake, arising from liver iron, of about 10 mg. daily. Excess hemoglobin production of about 100 gm. occurred. The same amount of inorganic iron (10 mg. per day) was added to the basal Cowgill ration for 2 weeks. No measurably increased hemoglobin regeneration was observed. Again the important factor contained in liver for hemoglobin regeneration does not appear to be contained in Lister casein. Casein also failed to facilitate hemoglobin regeneration after hemorrhage in McCay's experiments with dogs and rats (21).

The method of preparation (19) of Whipple's secondary anemia liver extract is such (the residue remaining after the pernicious anemia fraction is removed) that innumerable substances could be the important factor. The isolation of the active factor or factors contained in liver for hemoglobin regeneration in chronic hemorrhagic anemia could be accomplished by the use of additions to the basal ration of the many substances present in liver. Considering in Table I the much higher intake of copper in the liver period than in either the iron or the casein-iron periods, the simulation of the quantitative intake of copper simultaneously with that of iron in inorganic form to the intake present during a liver feeding period is the next obvious step. The addition of other types of protein, perhaps nucleoprotein, other minerals, hormones and vitamins (20) may perhaps be necessary to duplicate in this synthetic manner the hemoglobin response to liver feeding.

#### CONCLUSIONS

1. A group of dogs on a standard salmon bread diet with a slowly regenerating anemia were studied. The addition of liver to this diet during a 2 week period promoted a definitely greater regeneration of hemoglobin than did the addition of an amount of inorganic iron which was equivalent to that contained in the added liver. The more effective result attained with liver cannot, therefore, be attributed solely to the iron intake.

2. The greater response to liver is not due to its content of amino acids which are present in casein, since a diet containing an exactly

similar amount of calories, iron and protein nitrogen, made up of inorganic iron and casein does not cause a greater response than that obtained by the addition of that amount of inorganic iron alone to the standard basal diet.

3. Furthermore, the salmon bread diet does not produce a deficiency of the amino acids represented in casein, since dogs eating the high protein (casein) Cowgill dog ration show the same basal hemoglobin regeneration rate and a similar greater response to liver than to inorganic iron. The Cowgill ration, however, supplies some non-ferrous factor involved in hemoglobin regeneration which is not contained, to as great a degree at least, in the salmon bread.

4. Whipple's chronic hemorrhagic anemia of dogs serves as an accurate assay method for measuring the hemoglobin producing power of a substance. Quantitatively reproducible responses can be obtained.

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# A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

## II. A STUDY OF AN AZOPROTEIN-ANTIBODY SYSTEM\*

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The precipitin reaction may be considered the resultant of a series of competing bimolecular reactions, the quantitative outcome of which depends on the relative proportions in which the components are mixed (1). In a typical instance, the reaction between Type III pneumococcus specific polysaccharide and homologous antibody produced in the horse, it was shown that the entire course of the reaction could be expressed by simple equations derived from the mass law (1, 2). These equations permitted the quantitative expression of the behavior of an unknown serum over the entire reaction range with the aid of a small number of micro analyses for nitrogen.

Since the system studied above concerns hapten and antibody, it was considered desirable to test the new relationships on precipitin reactions between true antigens and their antibodies. Furthermore, in order that antigen could be directly determined in all precipitates and differentiated from the antibody thrown down at the same time, it was found convenient to make use of R-salt-azo-biphenyl-azo-crystalline egg albumin, a dark red antigen the preparation and properties of which have already been described (3). With this product antigen and antibody could be separately determined in the specific precipitates by the methods given in Reference 3, advantages which

\* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital. For Paper I of this series see Reference 1, which was published as Paper III of a series entitled, "The precipitin reaction between Type III pneumococcus polysaccharide and homologous antibody."

were not provided when colorless antigens were used. Preliminary data obtained in this way were published some years ago (4).

It is shown in the present communication that the theory outlined above (1) is applicable to the azoprotein-antibody system<sup>1</sup> with very little modification. An empirical relation is also given which permits the calculation of the maximum amount of specifically precipitable antibody from the same analyses used to establish the equation for the reaction in the region of excess antibody. Questions arising in connection with the determination of the excess of antigen in inhibition zone supernatants have also been studied.

### EXPERIMENTAL

Rabbit antisera were used in every instance and most of the sera were obtained<sup>2</sup> with the aid of multiple injections of 1 to 5 mg. of alum-precipitated dye (3). The sera were preserved with 0.01 per cent of merthiolate in order to avoid contamination during use.

The methods used for setting up the determinations and washing and analyzing the precipitates have been described in References 1 and 3. The colorimetric determination of the azoprotein in the specific precipitate appeared to be equally satisfactory whether or not colorless protein was added to the alkalized standard. Occasionally the color of the dissolved precipitate failed to match the standard, in which case light intensities were approximately matched. The optimum amount of alkali for both precipitates and standards in the colorimetric comparisons was about 0.1 cc. of normal sodium hydroxide per 5 cc. After the color estimation the solution of the precipitate was quantitatively rinsed into a micro Kjeldahl flask (100 cc. flasks were used) for the determination of total nitrogen. Total nitrogen minus antigen nitrogen equals antibody nitrogen. In general, nitrogen estimations are reported as far as the third decimal place, the exact value of which is, however, uncertain except in the case of total dye nitrogen added.

In Table I are given comparative data obtained at 37°C., at 0°, and by allowing the tubes to stand at room temperature for 1 or 2 hours and then in the ice box. In the experiments which were carried out at low temperatures throughout, the tubes were allowed to stand for 48 hours, and were stirred after 24 hours in the same way as described in References 1 and 3.

In Table II are given data obtained at low temperatures throughout. The serum was chilled in ice water before mixing with 1.0 cc. of the appropriate concentration of the dye solution and the tubes were usually allowed to stand in the ice box for 2 days. It was found, however, that this was not long enough for

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<sup>1</sup> Referred to subsequently as the dye-antidye system.

<sup>2</sup> This portion of the work was carried out by Mr. C. M. Soo Hoo.

experiments in the inhibition zone, in which a period of at least 4 days in the refrigerator was required before precipitation ceased. The results of this experiment are plotted in Text-fig. 1 as Lines A and B, and as Curve C, of which the last represents total nitrogen precipitated plotted against dye nitrogen precipitated.

Table III shows two parallel serial experiments on the supernatants from the first two pairs of tubes in the experiment summarized in Table II. Several serial experiments were run with larger amounts of other sera and also gave straight lines when the data were plotted as in Text-fig. 1.

In Table IV are given the maximum amounts of specifically precipitable nitrogen in many of the sera used in the present work. In each instance antigen and antibody were determined separately in the washed specific precipitate, and the figures given represent antibody nitrogen. The other data in Table IV are referred to in the discussion.

Table V gives data collected in the region of antigen excess, comprising the zone of maximum antibody precipitation and the inhibition zone.

The data summarized in Table VI deal with the determination of antigen in the supernatants of the inhibition zone by the specific precipitin method given in References 5 and 1. An aliquot portion of the supernatant was set up with fresh serum for which the total nitrogen curve, corresponding to C, Text-fig. 1, was known. Care was taken to choose a portion of the supernatant small enough to avoid complete exhaustion of the antibody added. The precipitate was analyzed for dye nitrogen and total nitrogen. The total nitrogen on the antibody curve corresponding to the dye nitrogen in the precipitate was then read from the curve and compared with the total nitrogen actually precipitated. It was thus possible to form some idea of the effect of the dissolved antibody in the inhibition zone supernatants on the analysis for antigen by this method. The results are discussed below.

#### *Supplementary Analytical Data on R-Salt-Azo-Biphenyl-Azo-Crystalline Egg Albumin (Cf. 3)*

The R-salt-azo-biphenyl-azo grouping,  $C_{22}H_{15}O_7N_4S_2$ , of formula weight 511.3, contains 12.54 per cent of S; egg albumin 1.57 per cent of S (6). The following micro analyses for S were run by Mr. William Saschek.

Azoprotein, dried to constant weight, 7.860, 11.815, 10.705, 11.130 mg.

BaSO<sub>4</sub>, 1.75, 2.20, 2.18, 2.27 mg.

S, 3.06, 2.56, 2.80, 2.81 per cent, calculated to the ash-free basis.

Calculated for azoprotein with 8 disazo groups; S, 2.78 per cent.

with 9 disazo groups, S, 2.91 per cent.

8 disazo groups are also equivalent to the coupling of one disazo group with each tyrosine group in egg albumin, which contains about 4.1 per cent of tyrosine (7).



## DISCUSSION

In the following discussion it is assumed that R-salt-azo-biphenyl-azo-crystalline egg albumin, fractionated according to Reference 3 and purified by ultrafiltration, can be treated as a single substance, also that the average behavior of the antidye is that of a single substance. The dye protein is almost entirely precipitable within very narrow limits of ammonium sulfate concentration, and practically all of the portion reactive in anti-egg albumin serum has been eliminated, but in spite of this the azoprotein may consist of a mixture of dyes, each containing

TABLE I  
*Precipitation of Antidye N at Various Temperatures*

| Dye N used | Dye N pptd.           | Antibody N pptd. | A:D ratio | Dye N pptd. | Antibody N pptd.      | A:D ratio | Difference in total N pptd. between 37° and 0° |
|------------|-----------------------|------------------|-----------|-------------|-----------------------|-----------|--|
| mg.        | mg.                   | mg.              |           | mg.         | mg.                   |           | mg.  |
|            | B 186 37° (2 hrs.)    |                  |           |             | B 186 0°              |           |  |
| 0.014(6)   | 0.013                 | 0.205            | 15.8      | 0.014(5)    | 0.223                 | 15.4      | 0.02   |
| 0.073      | 0.062                 | 0.636            | 10.3      | 0.068       | 0.742                 | 10.9      | 0.11   |
|            | B 189 II 37° (2 hrs.) |                  |           |             | B 189 II 0° (48 hrs.) |           |  |
| 0.093      | 0.069                 | 0.659            | 9.6       | 0.077       | 0.673                 | 8.7       | 0.02   |
|            | B 187 37° (2 hrs.)    |                  |           |             | B 187 20° and 0°*     |           |  |
| 0.029      | 0.025                 | 0.367            | 13.9      | 0.028       | 0.358                 | 12.8      | 0.01   |
| 0.058      | 0.049                 | 0.513            | 10.5      | 0.055       | 0.529                 | 9.6       | 0.02   |
| 0.088      | 0.069                 | 0.581            | 8.4       | 0.075       | 0.607                 | 8.1       | 0.03   |

A = antidye N, D = dye N.

\* At room temperature until precipitate began to separate, then in ice box 72 hours.

a different number of the R-salt-azo-biphenyl-azo groupings in the molecule. It is perhaps for this reason that in the region of excess antibody a given antidye serum precipitates only a certain proportion of the dye, characteristic for any serum, but varying from about 75 per cent to 95 per cent of the total added in different sera. From the sulfur content of the dye it would appear that the average number of disazo linkages in the product is eight or nine, figures consistent with Hooker and Boyd's finding that the larger atoxylazocasein molecule must contain at least thirteen azo groups in order to precipitate in the cross-reaction with atoxylazo egg white antiserum (8). It should be

recalled, also, that the egg albumin was not coupled with a maximal amount of the azo component (3), so that it is not surprising that the

TABLE II

*Addition of Increasing Amounts of Dye to 4.0 Cc. of Pooled Sera B 186, at 0°*

| Dye N added | Dye N pptd. | Total N pptd. | Anti-body N by difference | Ratio anti-body N:dye N in ppt. | Tests on supernatant       | Antibody N pptd., calcd. from equation [3] |
|-------------|-------------|---------------|---------------------------|---------------------------------|----------------------------|--|
| mg.         | mg.         | mg.           | mg.                       |                                 |                            | mg.  |
| 0.007(3)    | 0.007(2)    | 0.130         | 0.123                     | 17.1                            | Excess A                   | 0.102                                      |
| 0.014(6)    | 0.014(5)    | 0.238         | 0.223                     | 15.4                            | " "                        | 0.201                                      |
| 0.037       | 0.035       | 0.494         | 0.459                     | 13.1                            | " "                        | 0.445                                      |
| 0.048       | 0.047       | 0.604         | 0.557                     | 11.9                            | " "                        | 0.567                                      |
| 0.073       | 0.068       | 0.810         | 0.742                     | 10.9                            | " "                        | 0.743                                      |
| 0.098       | 0.091       | 0.962         | 0.871                     | 9.6                             | " "                        | 0.881                                      |
| 0.110       | 0.106       | 1.052         | 0.946                     | 8.9                             | No A or D                  | 0.941                                      |
| 0.146       | 0.134       | 1.168         | 1.034                     | 7.7                             | 0.002 mg. dye N in excess* | 0.988                                      |
| 0.183       | 0.162       | 1.242         | 1.080                     | 6.7                             | Excess D                   |  |
| 0.219       | 0.191       | 1.320         | 1.129                     | 5.9                             | " "                        |  |
| 0.256       | 0.207       | 1.318         | 1.111                     | 5.4                             | " "                        |  |
| 0.280†      | 0.229       | 1.360         | 1.131                     | 4.9                             | " "                        |  |
| 0.485       | 0.271       | 1.295         | 1.024                     | 3.8                             | " "‡                       |  |
| 0.970       | 0.249       | 0.931         | 0.682                     | 2.7                             | " "§                       |  |
| 1.455†      | 0.112       | 0.394         | 0.282                     | 2.5                             | " "                        |  |
| ‡           | 0.074       | 0.924         | 0.850                     | 11.5                            |                            |  |
| §           | 0.098       | 1.096         | 0.998                     | 10.2                            |                            |  |
| **          | 0.072       | 1.000         | 0.928                     | 12.9                            |                            |  |

Mean percentage of dye precipitated up to region of excess antigen, 95 per cent.

Maximum specific precipitable antibody N, calculated from Line B, Text-fig. 1, 1.098 mg.

\* Determined by precipitating 7.0 cc. of combined supernatants with 4.0 cc. B 186 and reading off dye from total N curve (cf. 5, 1).

† Not run in duplicate.

‡ 2.0 cc. portions run with 4.0 cc. B 186.

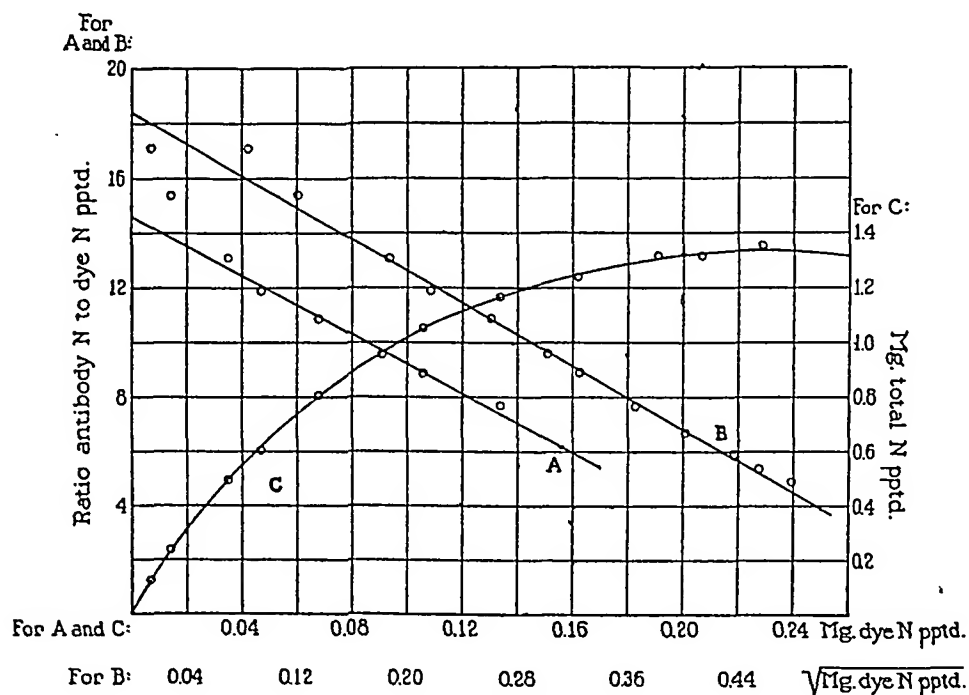
§ 0.70 cc. portions run with 4.0 cc. B 186, also \*\* 0.60 cc. portions.

amount combined corresponds roughly to the tyrosine content of the protein.

In planning the experiments it was necessary to ascertain the effect of changes in temperature on the amount of nitrogen specifically precipitable from antisera by the dye. Contrary to the effect noted in horse sera (9) there was in general only a slight difference in the amount and composition of the precipitate in the

experiments run at 37° and those carried out at 0°. It also appeared immaterial whether the runs were started at room temperature and continued at 0°, or were carried out entirely at low temperatures, as stated in Reference 3. The data on the influence of temperature are collected in Table I. The slight difference between the values at 37° and at 0°, roughly proportional to the quantity of precipitate, may be interpreted as an indication that these rabbit sera, unlike antipneumococcus horse sera, contain relatively little of an antibody fraction precipitable at 0° but not at 37°.

Experiments at pH 6.7 and 7.9 with antidye serum disclosed no differences, in agreement with Marrack and Smith (10), except possibly a slightly steeper



TEXT-FIG. 1

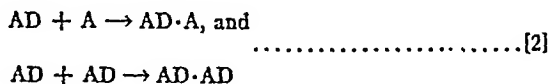
gradient in the inhibition zone at the higher pH, and are therefore not given in detail.

From the data summarized in Table II and given in graphic form in Text-fig. 1, it is evident that the quantitative theory of the precipitin reaction presented in Reference 1 is applicable to the present antigen-antibody system. The theory may be briefly restated as follows, but for the derivation and a detailed discussion the reader is referred to Reference 1.

The precipitin reaction between dye and antidye is considered as a series of bimolecular reactions which take place before precipitation occurs. The first step in the reaction between antidye (A) and dye protein (D) would then be



This would represent the equivalence point compound in its simplest form, as composed of 1 unit of A and 1 unit of D, regardless of their actual molecular proportions. Since both A and D are proteins, and the opportunity is given for the immunologically reactive groupings to recur a number of times, the reactants may be considered multivalent with respect to each other (*cf.* 1). Thus the AD compound initially formed could react with other molecules of the same compound, or with A or D, whichever is present in excess. In the region of excess antibody the second step of the reaction would then consist of the two competing bimolecular reactions in which dissociation is assumed to be negligible:



Since both A and D are multivalent with respect to each other the products of reaction [2] would combine chemically until aggregates large enough to separate from the solution are formed. If A and D are mixed in equivalent proportions the AD produced according to [1] would merely polymerize, and the equivalence point precipitate would be  $(AD)_n$ .

As in the reaction studied in Reference 1 the composition of the precipitate would thus depend on the relative proportions in which the reactants are mixed. The ratios given in Table II, and the colors of the precipitates, which range from pink to deep purplish red, are in accord with this view. Based on this concept of the reaction expressions [3] and [4] below may be derived with the aid of the mass law as in Reference 1. Since only a small proportion of the antibody was found to react to form compounds containing more A than twice the equivalence point ratio, the additional reactions which it was necessary

to consider in Reference 1 may be neglected. The equation, then, for reaction [2] in the region of excess antibody becomes:

$$\text{mg. antibody N precipitated} = 2 RD - \frac{R^2 D^2}{A} \dots\dots\dots [3]$$

in which R is taken as the ratio of antibody nitrogen to dye nitrogen at the point at which antigen first appears in excess,<sup>3</sup> D = the amount of dye nitrogen precipitated (= amount added  $\times$  a fraction characteristic for the serum), and A = the amount of antibody N precipitated at the point at which antigen first appears in excess.

In the region of excess D up to the inhibition zone the equation becomes:

$$\text{mg. dye N precipitated} = 2 R'A - \frac{(R')^2 A^2}{D \text{ added}} \dots\dots\dots [4]$$

Since the maximum amount of specifically precipitable nitrogen is not obtained at the equivalence point, but in the region of excess antigen R' is defined as  $\frac{1}{R}$ , or the ratio of dye nitrogen to antibody nitrogen in the precipitate at the point at which antigen first appears in excess, and A as the maximum specifically precipitable antibody nitrogen, D being the antigenic portion of the dye added (total  $\times$  a fraction characteristic for the serum used). Duplicate analyses with a slight excess of dye are sufficient to establish A.

In the inhibition zone an expression of the type given in Reference 2<sup>4</sup> is found to apply:

$$\frac{D_{\text{soln}}}{A_{\text{soln}}} = K \dots\dots\dots [5]$$

In this expression  $D_{\text{soln}}$  is the amount of antigenic dye nitrogen in solution and  $A_{\text{soln}}$  is the difference between A as defined for expression [4] and the amount precipitated at the point under consideration in the inhibition zone. While K is a constant for any serum over the

<sup>3</sup> Agreement of [3] with the experimental data is better if R is taken as the ratio at this point instead of at the equivalence point. The value of R is then not very different from the empirically useful R'' (see below).

<sup>4</sup> Heidelberger and Kendall (2), page 820.

greater part of the inhibition zone, it can scarcely be a true dissociation constant, since it is shown in Table II that the composition of the precipitate varies in this zone as well. Here, too, the relative proportions of the components appear to be the determining factors, since a diminution of the concentration of D by dilution with saline is not followed by precipitation, even after several days in the cold.

From expression [3] may be derived the linear relation:

$$\frac{\text{Antibody N}}{\text{Dye N}} \text{ in the precipitate} = 2R - \frac{R^2}{A} D \dots\dots\dots [6],$$

and in the text-figure Line A is plotted in this way from the data in Table II up to the region of excess antigen. Thus for the pooled sera B 186, the intercept on the y-axis,  $2R$ , = 14.6, and the slope of the line,  $\frac{R^2}{A}$ , = 54, whence  $R = 7.3$ ,  $A = 0.988$ . The experimentally found values are  $R = 7.7$ , at the antigen excess end of the equivalence zone, and  $A = 1.034$ . A comparison of the fourth and last columns of Table II shows close agreement between the experimentally determined values of antibody N precipitated and those calculated according to [3], except in the case of the first two points. Since [6] is a linear expression, the equation for the line may be approximately fixed for any serum by two determinations (in duplicate) of the amounts of antigen and antibody nitrogen precipitated in the region of excess antibody. Naturally, if more points are determined the accuracy is greater. More than one-third of the antibody should be precipitated, as the small portion present yielding compounds of ratio  $> 2R$  would otherwise introduce a relatively large error.

Data are given in Table III for experiments in which dye was added serially in small portions to an excess of B 186 mixture, using, in the two cases given, the supernatants from the initial precipitations with 0.007 and 0.015 mg. of dye N in the experiment summarized in Table II. The A:D ratios lie on a line somewhat above Line A in Text-fig. 1, as would be expected from the Danysz effect. Hence the amount of antibody N precipitated was actually higher for a given amount of antigen added in serial portions than calculated from Line A (equation [6]). This is shown by comparison of Columns 3 and 5 of Table III. Other serial experiments on larger quantities of serum

were also shown to follow expression [6], but the data were insufficient to permit a comparison at more than one point with the antibody N precipitated by a single addition of antigen. In one such experiment with the serum of Rabbit R 18, it was found that after a single addition of dye, 0.25 mg. of antibody N and 0.024 mg. of dye N were precipitated per cc., while practically the same amount of antibody N, 0.24, and only 0.016 mg. per cc. of dye N were precipitated if the dye was added serially in six small portions. The Danysz effect is thus brought within the theory presented.

TABLE III

*Serial Additions of Dye to Pooled Sera B 186, Calculated Back to 4.0 cc.*

| Total of successive dye N additions | Total dye N pptd. | Antibody N pptd. | Ratio A N:D N | Antibody N pptd., from equation [3] |   |
|-------------------------------------|-------------------|------------------|---------------|-------------------------------------|---|
| mg.                                 | mg.               | mg.              |               | mg.                                 |   |
| 0.014(6)                            | 0.014(5)          | 0.223            | 15.4          | 0.201                               |   |
| 0.037                               | 0.036             | 0.488            | 13.6          | 0.456                               |   |
| 0.059                               | 0.057             | 0.694            | 12.2          | 0.656                               |   |
| 0.087                               | 0.081             | 0.883            | 10.9          | 0.828                               |   |
| 0.116                               | 0.092             | 0.939            | 10.2          |                                     | Excess D; 0.19 mg antibody N not pptd.  |
| 0.007(3)                            | 0.007(2)          | 0.123            | 17.1          | 0.102                               |   |
| 0.029                               | 0.029             | 0.408*           | 14.1          | 0.378                               |   |
| 0.051                               | 0.051             | 0.623            | 12.2          | 0.605                               |   |
| 0.079                               | 0.077             | 0.842            | 10.9          | 0.804                               |   |
| 0.108                               | 0.093             | 0.936            | 9.9           |                                     | Excess D; 0.19 mg. antibody N not pptd. |

\* One determination lost.

Serial experiments at 0° in the dye-antidye system differed from those studied in Reference 1 in that a portion of the antibody was not precipitated, a circumstance which might contribute to the Danysz effect. Thus it is seen from Table III that about 17 per cent of the antibody present remained in solution, although this portion of the antibody was precipitated with the rest when enough dye was added in a single portion, as in Table II. It is possible that this part of the antibody contains too few specific groupings to build up A·D aggregates large enough to separate from solution unless these can combine

with A:D aggregates formed by the rest of the antibody. R 18, also, showed 13 per cent of antibody which was non-precipitable in a serial experiment, so that it is evident that even in serum from a single animal there are antibodies of different reactivities. The failure of the first two points in Text-fig. 1 to follow either linear relation has already given evidence that a portion of the antibody can react to form com-

TABLE IV

*Maximum Specifically Precipitable Nitrogen in Antidye Sera and Composition of Dye-Antidye Precipitate in Equivalence Zone and at Maximum Precipitation*

| Serum or antibody | Maximum specifically precipitable N found per cc. | Maximum calcd. from equation [7] | Ratio at maximum (= R*) calcd. from equation [7] | Ratio antibody N:dye N at beginning of equivalence zone | Equivalence point ratio | Ratio antibody N:dye N at end of zone (= R) | R calcd. from equation [6] |
|-------------------|---|----------------------------------|--|---|-------------------------|---|----------------------------|
|                   | mg.   | mg.                              |  |   |                         |   |                            |
| 7.42              | 0.11  |                                  |  | >13.2   | (>11.6)                 | (10)  |                            |
| 8.01              | 0.32  | 0.31 (4 points)                  | 6.7  | (<12)   | (<10.2)                 | 8.4   | 7.8                        |
| 8.08              | 0.14  | 0.14 (2 " )                      |  | >12.9   | (>11.5)                 | (10)  |                            |
| R 18              | 0.27  | 0.28 (2 " )                      |  | >10.4   | (>9.2)                  | >8.0  |                            |
| 1.14              | 0.51  | 0.52 (2 " )                      |  |   |                         | 8.3   |                            |
| 1.15 + 1.46*      | 0.27  | 0.28 (3 " )                      | 6.4  |   |                         | 8.3   |                            |
| CV18 serum        | 0.29  | 0.30 (2 " )                      |  |   |                         | (7.0)                                       |                            |
| CV18 globulin     | 0.19  | 0.19 (3 " )                      | 6.0  | (10)  | (8.3)                   | 6.6   | 6.9                        |
| B 186*            | 0.28  | 0.27 (12 " )                     | 6.1  | (9.4)   | (8.6)                   | 7.7   | 7.3                        |
| B 187*            | 0.16  | 0.16 (8 " )                      | 6.8  | (10.5)  | (8.6)                   | 6.6   | 8.1                        |
| 3.76              | 0.26  | 0.26 (5 " )                      | 6.1  | 9.9   | (9.0)                   | 8.1   | 7.3                        |
| Mean.....         |   |                                  | 6.4  |   |                         |   |                            |

Values in parentheses indicate probable value deduced from nearest actual determination.

\* Pooled sera.

pounds of greater A:D ratio than 2 R. It is not surprising, therefore, that there are experimental deviations from a theory based on the statistical treatment of the antibody as a unit.

In most instances insufficient experiments were run to fix the equivalence zone with great precision, but in the last four sera (two of which were mixtures) (see Table IV) it was quite accurately mapped out. These sera showed an average calculated equivalence point



of 8.6, with considerable variation in the extent of the zone. It must be pointed out, however, that at least two sera, 7.42 and 8.08, must have had equivalence point ratios exceeding 10 (see also Table IV). A more detailed discussion of questions related to the equivalence zone will be given in connection with data on the crystalline egg albumin-antibody system.

TABLE V  
*Comparison of Calculated and Found Values of Dye Precipitated in Region of Antigen Excess, B 186*

| Dye N added<br>(95% antigenic) | Antigenic dye N added | Dye N pptd.<br>(found) | Dye N pptd., from equation [4]                              |   |
|--------------------------------|-----------------------|------------------------|---|---|
|                                |                       |                        | Using experimental values $A = 1.13$ , $R' = \frac{1}{7.7}$ | Using values calcd. from equation [7] $A = 1.10$ , $R' = \frac{1}{6.1}$ |
| mg.                            | mg.                   | mg.                    | mg.   | mg.   |
| 0.146                          | 0.139                 | 0.134                  | 0.139   | 0.127   |
| 0.183                          | 0.174                 | 0.162                  | 0.170   | 0.174   |
| 0.219                          | 0.208                 | 0.191                  | 0.190   | 0.205   |
| 0.256                          | 0.243                 | 0.207                  | 0.205   | 0.227   |
| 0.280                          | 0.266                 | 0.229                  | 0.213   | 0.239   |

*Inhibition Zone*

|       |       |       | Dye N in solution | Antibody N dissolved |           | K, equation [5] |           | Antibody N dissolved (calcd.) |          |
|-------|-------|-------|-------------------|----------------------|-----------|-----------------|-----------|-------------------------------|----------|
|       |       |       |                   | A = 1.131            | A = 1.098 | A = 1.131       | A = 1.098 | K = 1.50                      | K = 1.59 |
| 0.485 | 0.461 | 0.271 | 0.190             | 0.107                | 0.074     | 1.78            | 2.57      | 0.127                         | 0.120    |
| 0.970 | 0.921 | 0.249 | 0.672             | 0.449                | 0.416     | 1.50            | 1.62      | 0.448                         | 0.423    |
| 1.455 | 1.383 | 0.112 | 1.271             | 0.849                | 0.816     | 1.50            | 1.56      | 0.847                         | 0.799    |

Columns 1 and 3 are taken from Table II.

When the maximum amount of precipitable antibody nitrogen in an antidye serum has been determined, or else has been calculated according to the method given in the following paragraph, the amount of dye nitrogen precipitated in the region of excess antigen up to the inhibition zone may be computed for various additions of dye by means of equation [4]. Calculated and found values for pooled sera B 186 are compared in Table V, making use of the experimentally determined figures for A and R' on the one hand, and the values calculated according to the next paragraph, on the other. It will be seen

that the agreement is good, particularly when the experimental values of A and R' are used.

By means of an empirical relation the same two or three points determined by chemical analysis in the region of excess antibody may be used to calculate the maximum amount of antibody nitrogen precipitable from the serum if it is desired to omit separate analyses to determine this constant. It has been found that if  $\frac{\text{Antibody N}}{\text{Dye N}}$  in the precipitate is plotted against the square root of the dye nitrogen precipitated an even closer approximation to a straight line is obtained than when the points determined are plotted according to equation [6]. For the six sera for which sufficient data are available this empirical relation has been found to reduce to the general form:

$$\frac{\text{Antibody N}}{D} \text{ in the precipitate} = 3 R'' - 2 \sqrt{\frac{(R'')^2 D}{A}} \dots\dots\dots [7]$$

in which  $3 R''$  is the intercept on the y-axis and  $- 2 \sqrt{\frac{(R'')^2}{A}}$  is the slope of the line,  $D$  = amount of dye nitrogen precipitated,  $A$  = maximum precipitable antibody nitrogen, and  $R''$  the A:D ratio at the maximum. For B 186 the equation for this line, drawn through the points experimentally determined for 4 cc. of serum (Line B, Text-fig. 1) is  $\frac{\text{Antibody N}}{\text{Dye N}}$  in the precipitate =  $18.4 - 29\sqrt{D}$ . Then antibody N precipitated =  $18.4 D - 29 D^{3/2}$ , and when the first derivative,  $18.4 - 43.5 D^{1/2} = 0$ , antibody N precipitated at the maximum, or  $A$ , = 1.10, a value in good agreement with that, 1.13, actually found. Found and calculated values for A are given in Table IV for ten sera, and it will be seen that the greatest deviation is 0.01 mg. of N per cc., a result well within the experimental error of the analytical methods used and more accurate than obtainable from [6] with only two sets of analyses. By this method, then, constants A and  $R''$  characteristic of the serum are obtained, the former, at least, with a high degree of accuracy. The calculated values for  $R''$  given in Table IV are quite uniform and in reasonable agreement with the experimental ratios, for it must be borne in mind that experimentally it is found that maximum precipitation often occurs over a fairly wide range of antigen ex-

cess, and unless a large number of analyses are made it is difficult to locate exactly the smallest amount of D yielding the A value. Thus, in B 186, maximum values for A were obtained at antibody N:D ratios from 5.9 to 4.9 (Table II), the former being in good agreement with the calculated value. The exact mapping out of this zone would scarcely be necessary for ordinary purposes, and equation [7] may be

TABLE VI  
*Analyses of Inhibition Zone Supernatants for Antigen*

| Antigenic dye N added                          | Antigenic dye N in 5.0 cc. supernatant | Antibody N in 5.0 cc. supernatant | Fraction of supernatant analyzed | Antigenic dye N in aliquot taken (from Column 2) | Dye N found colorimetrically in aliquot taken | Total N on Curve C, Text-fig. 1 corresponding to D found | Total N actually determined | Difference | Antibody N in aliquot of inhibition supernatant |
|--|--|-----------------------------------|----------------------------------|--|---|--|-----------------------------|------------|---|
| mg.  | mg.                                    | mg.                               |                                  | mg.  | mg.   | mg.  | mg.                         | mg.        | mg.   |
| B 186  |  |                                   |                                  |  |   |  |                             |            |   |
| 0.461  | 0.190                                  | 0.107                             | 0.4                              | 0.076  | 0.074   | 0.850  | 0.92                        | 0.07       | 0.04  |
| 0.921  | 0.672                                  | 0.449                             | 0.14                             | 0.094  | 0.098   | 1.00   | 1.10                        | 0.10       | 0.06  |
| 0.921  | 0.672                                  | 0.449                             | 0.12                             | 0.081  | 0.072*  | 0.84   | 1.00                        | 0.16       | 0.05  |
| B 187 (total N curve not given in Text-fig. 1) |  |                                   |                                  |  |   |  |                             |            |   |
| 0.128  | 0.016                                  | 0.012                             | 0.8                              | 0.013  | Not run                                       | 0.210†   | 0.238                       | 0.03       | 0.01  |
| 0.257  | 0.147†                                 | 0.150                             | 0.2†                             | 0.029  | 0.025   | 0.295†   | 0.350†                      | 0.055      | 0.03  |
| 0.411  | 0.314                                  | 0.283                             | 0.133                            | 0.042  | 0.039   | 0.494  | 0.570                       | 0.08       | 0.04  |
| B 189 (total N curve not given in Text-fig. 1) |  |                                   |                                  |  |   |  |                             |            |   |
|  | In 2.5 cc. supernatant                 |                                   |                                  |  |   |  |                             |            |   |
| 0.149  | 0.068                                  | 0.01                              | 0.4                              | 0.027  | 0.027   | 0.310  | 0.350                       | 0.04       | 0.00  |
| 0.299  | 0.207                                  | 0.133                             | 0.2                              | 0.041  | 0.042   | 0.398  | 0.420                       | 0.02       | 0.03  |

\* Poor color match.

† Corresponding to calculated amount taken.

‡ Using B 189, which precipitated 80 per cent of dye added. 1.5 cc. portions B 189 used.

used as far as the inhibition zone when the minimum number of analyses seems desirable. The resulting error beyond the maximum did not exceed 10 per cent in the sera studied.

It remains, then, only to determine the amount of antibody and antigen nitrogen dissolved at a point well within the inhibition zone to obtain a value of K in equation [5], and when this is found, the

amount and composition of the precipitate in this range may be calculated. Here, too, it would be advantageous to perform analyses at two points. Inhibition zone data for B 186 are given in the second part of Table V. In the case of B 187 the values of  $K$  found from Columns 2 and 3 of Table VI according to equation [5] were 1.08 and 1.11.

Thus with a minimum of three sets of micro analyses for dye and antidye nitrogen precipitated, two in the region of excess antibody, and one in the inhibition zone, it is possible to derive expressions from which the amount of antibody nitrogen precipitated by any amount of antigen may be roughly calculated, as may also be the amount of antigen precipitated in the maximum antibody zone. With a larger number of analyses the entire behavior of the serum in the precipitin reaction may be predicted with a satisfactory approximation to the experimentally determined values.

The above considerations have been presented with the use of a single serum mixture, B 186, as the principal example. Less complete data are available with other sera, and in some of these agreement with the theory is even more satisfactory than in the case of B 186. In order to save space most of these data have been omitted, the more so as the utility of the quantitative precipitin theory in the case of a pure, crystalline, colorless antigen, egg albumin, and its homologous antibody has been demonstrated and the data are being prepared for publication.

The dye-antidye system is thus chiefly of use in permitting a series of orientating experiments which would not have been possible in a system with a colorless antigen. As an additional example, the determination of the excess of antigen in the supernatant in the region between the equivalence zone and the inhibition zone according to Reference 1 or 5 offers no complications with either a colored or colorless antigen. In the latter case, the amount of antigen in solution in the inhibition zone can be determined from an aliquot portion of the supernatant, set up against fresh antibody in excess, only after the effect of the dissolved antigen-antibody compound present is first ascertained. Since there is no way of distinguishing between antigen nitrogen and antibody nitrogen in a colorless precipitate or supernatant it was necessary to study the question in the dye-antidye

system. The data so obtained are summarized in Table VI, in which the amount of antigenic dye N and antibody N in the supernatant in the case of B 186 are taken from Table V, using  $A = 1.13$  as the maximum. It would seem that the entire amount of dissolved antigen-antibody compound present in the aliquot appears in the precipitate formed with fresh antibody in excess, a result which might have been predicted on the basis of the writers' theory. A small, but consistent discrepancy in these determinations is also evident, averaging 0.03 or 0.04 mg. of nitrogen. This appears even in the instance in which no appreciable amount of soluble AD compound had been formed, and may be due to the presence of a portion of the difficultly precipitable antibody mentioned in connection with the Danysz effect.

Since the writers' preliminary work (4) studies on azoprotein-antibody systems have been made by Marrack and Smith (10) and by Haurowitz and Breinl (11). Both groups confirmed the varying composition of the specific precipitate with varying proportions of antigen and antibody.

In conclusion it must again be emphasized that the present theory represents an attempt to deal formally with antigen and antibody on the basis that these behave statistically as multivalent, homogeneous substances. There is ample evidence that antibody, in the systems already studied, is a mixture of substances of different reactivities, so that the present quantitative theory is offered merely as a useful expedient, applicable to antisera as they occur, until such time as it may be possible to separate from the complex antibody mixture an antibody possessed of a single reactivity.

#### SUMMARY

1. A quantitative theory of the precipitin reaction based on the laws of classical chemistry has been tested on an azoprotein-antiprotein system and found to apply.

2. With its aid relationships may be deduced which permit the calculation of the behavior of an antidye serum over its entire range after a few quantitative chemical analyses have been made for antigen and antibody in the precipitate.

3. An empirical relation is also presented which further reduces the number of analyses necessary.

4. A study of supernatants in the inhibition zone has shown that the entire amount of dissolved antigen-antibody compound present is precipitated when supernatants are analyzed for antigen by the precipitin method.

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## LIPOIDS AND IMMUNOLOGICAL REACTIONS

### I. THE RELATION OF PHOSPHOLIPINS TO THE TYPE-SPECIFIC REACTIONS OF ANTIPNEUMOCOCCUS HORSE AND RABBIT SERA

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It has been recognized that the antibacterial sera produced by the immunization of the horse and the rabbit with Type I pneumococci, although possessing type specificity, exhibit marked differences in certain immunological properties. The distinctive characteristics of the two sera have interested numerous investigators, but as yet there is almost no evidence to indicate what constituents of the two sera are responsible for the differentiating qualities.

The precipitates formed by the inter-reaction of type specific antipneumococcus antibodies and specific capsular polysaccharide can be differentiated by their *macroscopic* characteristics, which are dependent upon the species by which the antibody is produced. For example, the precipitate with immune horse serum is a granular opaque cake, while that produced in a similar manner with immune rabbit serum is a thin translucent disc. Schiemann and Casper (1) have shown that these immune precipitates form characteristic *microscopic* pictures which can be distinguished one from the other. Zinsser and Parker (2) have demonstrated that antipneumococcus horse serum and homologous pneumococci, or the specific soluble substance, do not bind complement, although with antipneumococcus rabbit serum complement is fixed. Avery and Tillett (3) have shown that antipneumococcus horse serum does not passively sensitize guinea pigs to the homologous capsular polysaccharide, while after the injection of antipneumococcus rabbit serum these animals can be shocked anaphylactically with the specific capsular polysaccharide. Etinger-Tulczynska (4) states that antipneumococcus rabbit serum constantly causes the phenomenon of *Quellung* with homologous pneumococci, while antipneumococcus horse serum does so very irregularly. In a previous communication (5) it has been shown that antipneumococcus horse serum must be used in definitely limited quantities to protect mice against large numbers of homologous pneumococci. It has also been demonstrated that when this optimum protective quantity of horse serum is exceeded, protective action is progressively diminished until with large amounts of serum



protection is completely abolished. This phenomenon has been termed the "prozone." Antipneumococcus rabbit serum, on the other hand, does not exhibit a similar result, but protects when comparable quantities are used. In confirmation of the prozone phenomenon with immune horse serum, the protocols of other workers also demonstrate the failure of greater than optimal amounts of horse serum to protect (Yosioka (6), Felton (7) and Sobotka and Friedländer (8)). It has been repeatedly shown by Felton (9) that the immune globulin fraction of antipneumococcus horse serum is precipitated by tenfold dilution with distilled water. However, the immune globulin fraction of antipneumococcus rabbit serum remains in solution after similar water dilution.

The distinguishing characteristics of sera produced by the immunization of animals of different species with the same bacterial cells are presented in Table I.

TABLE I

*The Distinguishing Characteristics of Horse and Rabbit Type I Antipneumococcus Sera*

|  | Type I antipneumococcus horse serum | Type I antipneumococcus rabbit serum |
|--|-------------------------------------|--------------------------------------|
| Precipitate with SSS* . . . . .  | Granular cake                       | Translucent disc                     |
| Complement fixation with SSS . . . . .                                     | —                                   | +                                    |
| Passive sensitization of guinea pig to SSS . . . . .                       | —                                   | +                                    |
| Neufeld's <i>Quellung</i> phenomenon . . . . .                             | ±                                   | +                                    |
| Prozone phenomenon . . . . .   | +                                   | —                                    |
| Precipitation of immune fraction by 9 volumes of distilled water . . . . . | +                                   | —                                    |

\* SSS indicates acetyl polysaccharide of Type I Pneumococcus.

Although the fraction of antipneumococcus horse serum which contains the type specific antibodies has been extensively studied, particularly with regard to the so called immune protein, relatively little investigation has been directed toward the lipoids of the serum. Felton and Kauffmann (10) have shown that the sera of horses immunized with Type I or Type II Pneumococcus contain, on the average, 27 per cent more lipid than does normal horse serum. They also state that the extraction of lipoids from antipneumococcus horse serum, by means of alcohol and ether in the cold, does not alter the protective action of the serum.

The present investigation was undertaken with the purpose of defin-

ing the rôle of lipoids in the immunological reactions of antipneumococcus serum. It was also desired to learn what relation lipoids might bear to the distinctive differences between antipneumococcus sera produced by the horse and the rabbit.

The simplest means by which the significance of the lipid fraction of antipneumococcus serum can be studied is to compare the immunological reactions of serum before and after the extraction of lipoids. This method has been applied to other immune sera by previous investigators. Hardy and Gardiner (11) removed lipoids from diphtheria antitoxin by means of alcohol and ether in the cold and showed that the lipid-free globulin became soluble in saturated NaCl solution. They state that the antitoxic properties of the serum were not changed by extraction. Hartley (12) extracted lipoids by a similar method from a large number of antisera, including rabbit anti-horse, rabbit anti-human, horse antityphoid, and horse diphtheria antitoxin. He was able to demonstrate a loss of precipitating ability in the extracted antiprotein sera, but found that diphtheria antitoxin and antityphoid sera still gave the usual characteristic reactions.

#### EXPERIMENTAL

*Methods of Lipoid Extraction.*—Two procedures have been used for the removal of lipoids from the immune sera. These were so designed as to permit of thorough extraction by means of lipid solvents and quantitative recovery of undenatured protein.

(A) *Alcohol and Ether Extraction.*—The following method, somewhat modified from that used by Hartley (12), was employed. Antipneumococcus serum was introduced drop by drop, with stirring, into ten volumes of absolute ethyl alcohol previously cooled to  $-10^{\circ}\text{C}$ . The centrifuge tubes containing the alcohol and the precipitated serum were frequently and vigorously shaken, and the temperature was constantly kept below  $-5^{\circ}\text{C}$ . After 6 hours extraction the precipitate was collected by rapid centrifugation at a temperature below  $-2^{\circ}\text{C}$ ., the supernatant liquid was decanted, and the precipitate again extracted at  $-10^{\circ}\text{C}$ . with a quantity of absolute alcohol equal to that first used. After 12 hours extraction the precipitate, collected in the same manner as before, was extracted with anhydrous ether for 10 hours at  $-10^{\circ}\text{C}$ ., and for a second time with anhydrous ether for 10 hours at room temperature. The precipitate was packed by centrifugation, the supernatant liquid decanted, and the precipitate freed of the last traces of ether by vacuum distillation. The resulting dry residue was dissolved in a quantity of saline equal to the original serum volume.

(B) *Alcohol, Petroleum Ether and Ether Extraction.*—Antipneumococcus serum

was treated in a manner identical to that described in Method A, until the second extraction with absolute alcohol had been completed. The precipitate was then extracted with low boiling point (40–60°C.) petroleum ether for 24 hours at –5°C. The precipitate was packed by centrifugation and extracted with anhydrous ether exactly as in Method A.

The precipitates recovered after these extractive procedures form perfectly clear solutions in 0.9 per cent saline. In physical appearance these solutions do not differ materially from the original sera.

It will be observed that certain definite temperatures were maintained at the various stages of the extractive methods. These are of importance for two reasons: (a) If the temperature of the solvents is allowed to exceed certain limits the proteins are rapidly denatured, and (b) if the temperature is maintained below a certain point the extraction of lipoids is considerably retarded.

#### *Properties of Type I Antipneumococcus Horse Serum after Extraction of Lipoids*

Type I antipneumococcus horse serum which had been extracted with alcohol and ether in the cold by Method A was studied by means of the usual *in vitro* and *in vivo* tests to determine what changes, if any, had been produced in the immunological characteristics of the serum as a result of the extraction of lipoids. Quantitative determinations before and after extraction showed a loss of 92 per cent of serum lipoids. In confirmation of the early work of Hoppe-Seyler (13), and that of numerous others, it was found that ether extraction alone removed only a relatively small proportion of total lipoids from immune serum. It was also found that ether extraction, or alcohol and ether extraction, of dried serum was inadequate and removed only a moderate proportion of total serum lipoids. The results of agglutination and precipitation tests with immune horse serum before and after the extraction of lipoids are presented in Table II.

Although the unextracted immune horse serum, at a dilution of 1:80, agglutinated a suspension of Type I pneumococci, no visible agglutination could be detected when extracted serum was mixed with either living or heat-killed homologous pneumococci. At a dilution of 1:4,000,000 the specific capsular polysaccharide forms a definite precipitate with the untreated serum. However, the extracted serum does not form any visible precipitate with the capsular polysaccharide in the concentrations used.

The results presented in Table II indicate that the lipoids of antipneumococcus horse serum are essential to the visible manifestations of the reactions of type specific agglutination and precipitation. This interpretation of the results of the *in vitro* tests is tenable if it is possible to show that some specific immune property, as, for example, protective action, remains unaltered in the extracted serum, since it

TABLE II

*Agglutination of Type I Pneumococci, and Precipitation with Capsular Polysaccharide by Type I Antipneumococcus Horse Serum, before and after Extraction of Lipoids*

|   | Serum dilution                 | Type I antipneumococcus horse serum |  |
|---|--------------------------------|-------------------------------------|--|
|   |                                | Before extraction Lot 4             | After extraction (Method A) Lot 4 (A.E.) |
| Agglutination of Type I Pneumococcus              | 1:10                           | ++++*                               | -†                                       |
|   | 1:20                           | ++++                                | -  |
|   | 1:40                           | +++                                 | -  |
|   | 1:80                           | +++                                 | -  |
|   | 1:160                          | -                                   | -  |
|   | Acetyl polysaccharide dilution |                                     |  |
| Precipitation with Type I capsular polysaccharide | 1:10,000                       | ++++                                | -†                                       |
|   | 1:40,000                       | ++++                                | -  |
|   | 1:400,000                      | ++++                                | -  |
|   | 1:4,000,000                    | ++                                  | -  |

\* Readings after 2 hours at 37°C. and 18 hours at 0°C.

† No evidence of visible agglutination or precipitation, although in these tubes there was a slight opalescence.

is obvious that the antibody might have been destroyed completely by the extractive procedure. The results of protection tests in mice with immune horse serum, before and after the extraction of lipoids, are shown in Table III.

It will be observed that after the extraction of lipoids from the serum, mice were protected against fatal infection with Type I Pneumococcus. The protective titers of the serum before and after extraction were identical, in that 0.05 cc. amounts in both instances protected against  $10^{-2}$  cc. of culture.

It is evident, therefore, that although the extraction of lipoids caused a loss of type specific agglutinating and precipitating properties, the capacity of the extracted serum to protect mice against infection remained entirely unaltered.

Perhaps the most significant peculiarity of antipneumococcus horse serum is the prozone phenomenon, as evidenced by the failure of greater than optimum amounts of serum to protect mice against fatal infection with homologous pneumococci. It is important to the definition of the significance of the changes produced in horse serum

TABLE III

*Protection Tests with Type I Antipneumococcus Horse Serum before and after Extraction of Lipoids*

| Type I pneumococcus culture | Type I antipneumococcus horse serum |          |   |          | Virulence control |
|-----------------------------|-------------------------------------|----------|---|----------|-------------------|
|                             | Before extraction<br>Lot 4          |          | After extraction<br>(Method A) Lot 4 (A.E.) |          | No serum          |
|                             | 0.10 cc.                            | 0.05 cc. | 0.20 cc.                                    | 0.05 cc. |                   |
| cc.                         |                                     |          |   |          |                   |
| $10^{-2}$                   | S                                   | S        | S   | S        | —                 |
| $10^{-3}$                   | S                                   | S        | S   | S        | —                 |
| $10^{-4}$                   | S                                   | S        | S   | S        | —                 |
| $10^{-5}$                   | S                                   | S        | S   | S        | —                 |
| $10^{-6}$                   | S                                   | —        | S   | —        | D 44              |
| $10^{-7}$                   | —                                   | —        | —   | —        | D 64              |
| $10^{-8}$                   | —                                   | —        | —   | —        | D 46              |

S = survival.

D = death at indicated number of hours.

after the extraction of lipoids, to determine what effect their absence might have upon this protective prozone. In Table IV the results of a protection test designed to illustrate the prozone are given.

The optimum protective quantity of antipneumococcus horse serum was not changed by the extraction of lipoids by Method A, as is evidenced by the uniform protection resulting when 0.1 cc. of either untreated or extracted serum was injected with 0.05 cc. of culture. The prozone phenomenon is as readily demonstrable in the extracted as in the unextracted serum, since in both instances with greater than optimal amounts of serum the protective action rapidly decreases until with 0.4 cc. of serum protection is completely lost.

From the results of protection tests shown in Table IV, it is apparent that after extraction of lipoids from antipneumococcus horse serum the prozone phenomenon was still demonstrable as manifested by the failure of larger amounts of the extracted serum to protect mice against fatal infection with homologous pneumococci. Under the conditions of this experiment it may be stated that the extraction of lipoids from immune horse serum does not alter the prozoning action of the serum when used in excess.

The results of the preceding experiments may be summarized by stating that although the removal of lipoids from antipneumococcus horse serum resulted in a loss of agglutinating and precipitating prop-

TABLE IV

*Protection Tests with Increasing Amounts of the Same Lot of Type I Antipneumococcus Horse Serum before and after Extraction of Lipoids, Illustrating the Prozone Phenomenon*

| Amount of antiserum | Type I antipneumococcus horse serum |      |   |      |
|---------------------|-------------------------------------|------|---|------|
|                     | Before extraction<br>Lot 4          |      | After extraction<br>(Method A) Lot 4 (A.E.) |      |
| cc.                 |                                     |      |   |      |
| 0.4                 | D 22                                | D 32 | D 22  | D 22 |
| 0.2                 | D 24                                | D 24 | D 22  | S    |
| 0.1                 | S                                   | S    | S   | S    |
| 0.05                | S                                   | S    | S   | S    |

All mice received 0.05 cc. of Type I pneumococcus culture, of virulence such that  $10^{-8}$  cc. killed mice in 48 hours.

erties *in vitro*, the protective action of the extracted serum *in vivo* remained quantitatively unchanged. The clue to the nature of these paradoxical results was found in studies of the mechanism of protection in mice which had been injected with culture and extracted serum. Although *in vitro* no agglutination had been visible, it was found that, when the extracted serum was injected together with pneumococci into the peritoneum of the mouse, agglutination occurred *in vivo* in a manner comparable to that in controls in which unextracted serum had been used.

Inasmuch as extracted horse serum protected mice against fatal infection equally as well as untreated horse serum, it seemed not

impossible that upon injection into mice the extracted serum might have been restored to a state similar to that existing before the extraction of lipoids. This hypothesis predicates the presence of the necessary lipid substances within the mouse peritoneum, and also the ability of the extracted serum to become reassociated with these lipoids. If both these assumptions are correct, the extracted serum should, after injection, regain its type specific agglutinating and precipitating capacities when withdrawn from the peritoneum and retested *in vitro*.

To test this hypothesis, Type I antipneumococcus horse serum from which the lipoids had been extracted was injected intraperitoneally into mice, and after 30 minutes the peritoneal fluid was withdrawn by means of a capillary pipette. This fluid was found to be capable of agglutinating Type I pneumococci, and of precipitating with the specific capsular polysaccharide, although prior to injection the extracted serum showed no visible evidence of these reactions.

This experiment indicates that although the extraction of lipoids from antipneumococcus horse serum causes the loss of certain *in vitro* reactions, this change is not irreversible, and that under certain circumstances it is possible to restore the type specific agglutinating and precipitating properties to the extracted serum.

With the purpose of determining what effect the extraction of lipoids from antipneumococcus horse serum had upon certain other distinctive qualities of the unextracted serum, the extracted serum was tested for capacity to fix complement, and also for the precipitability of the immune globulin fraction upon water dilution.

It was found that the immune horse serum, after extraction of lipoids, did not fix complement with homologous pneumococci, or with the specific capsular polysaccharide, a property likewise characteristic of unextracted serum.

On tenfold dilution with distilled water, it was evident that the solubility of the globulin fraction of the extracted horse serum had been markedly changed, for almost no precipitate formed.

When 0.5 cc. of extracted serum was diluted with 4.5 cc. of distilled water in a microtip centrifuge tube, allowed to stand in the ice box overnight, and the solution centrifuged for  $\frac{1}{2}$  hour, the packed precipitate amounted to but 0.001 cc. When 0.5 cc. of unextracted antipneumococcus horse serum was carried through

the identical procedure, the packed precipitate equalled 0.06 cc., or 60 times the volume of the precipitate from extracted serum.

These results tend to show that the solubility of the immune globulin of antipneumococcus horse serum is considerably modified by the lipid constituents of the serum. Although a large proportion of the immune globulin of untreated serum is precipitated by tenfold dilution with water, almost none is precipitated by this means after the removal of lipoids. Furthermore, the protective antibody of the extracted serum remains almost quantitatively in the supernatant liquid after tenfold water dilution.

*Absorption of Protective Antibody from Extracted Type I  
Antipneumococcus Horse Serum*

The results of the preceding experiments have demonstrated that the extraction of lipoids from Type I antipneumococcus horse serum results in an apparent loss of *in vitro* activity, as evidenced by the lack of specific precipitation and agglutination. However, since the extracted serum protected mice as well as did unextracted serum, it became of interest to determine whether any binding of antibody by the pneumococcus or its capsular polysaccharide occurred *in vitro* even in the absence of the physical changes which usually denote antigen-antibody union.

In studying this phase of the problem, two separate types of absorption experiments were performed. In the first, 4.0 cc. of antipneumococcus horse serum, after the extraction of lipoids, was diluted with 6.0 cc. of saline and mixed with the living pneumococci centrifuged from 250 cc. of an 18 hour broth culture. After incubation at 37°C. for 2 hours, and 18 hours in the ice chest, the mixture was centrifuged for 1 hour. The supernatant fluid was again absorbed in a similar way and finally was passed through a Berkefeld V filter. The second method differed from the first in that in this instance absorption was carried out with bacterial cells which had been heat-killed, and subsequently washed four separate times with 60 cc. of saline. After each washing the pneumococci were recovered by centrifugation for 1 hour. 2.0 cc. of extracted serum were mixed with organisms prepared in this way from 150 cc. of broth culture of Type I Pneumococcus, the mixture was incubated for 2 hours at 37°C. and kept in the ice chest overnight. The supernatant fluid was pipetted off after centrifugation for 1 hour and again absorbed in a similar way. In both methods an equal volume of unextracted serum was treated in an identical manner.



Following absorption with pneumococci by the above two methods, protection tests were carried out with both the extracted and the unextracted sera. The results of these experiments are presented in Table V.

The results of protection tests with extracted horse serum, which had been absorbed with pneumococci, are quite different from the results of tests with unextracted serum similarly absorbed (Table V). With unextracted serum, absorption removed all demonstrable protective antibody even when repeatedly washed heat-killed organisms were used. With extracted serum, however, an appreciable amount

TABLE V

*Comparative Results of Protection Tests with Whole and Extracted Type I Anti-pneumococcus Horse Serum, after Absorption with Type I Pneumococci*

| Type I pneu-<br>mococcus<br>culture | Type I antipneumococcus horse serum (0.2 cc.) |   |                                  |        |   |         |
|-------------------------------------|---|---|----------------------------------|--------|---|---------|
|                                     | Before<br>extraction<br>Lot 4                 | After<br>extraction<br>(Method A)<br>Lot 4 (A.E.) | Before extraction<br>Lot 4       |        | After extraction<br>(Method A) Lot 4 (A.E.) |         |
|                                     | Unabsorbed                                    |   | Absorbed with Type I pneumococci |        |   |         |
|                                     |   |   | Living                           | Washed | Living                                      | Washed  |
| cc.                                 |   |   |                                  |        |   |         |
| 10 <sup>-2</sup>                    | S S S D                                       | S S S S   | —                                | —      | —   | —       |
| 10 <sup>-3</sup>                    | S S S D                                       | S S S S   | D D                              | D D    | D D D D                                     | D D D D |
| 10 <sup>-4</sup>                    | S S   | —   | D D                              | D D    | D D D D                                     | D D S S |
| 10 <sup>-5</sup>                    | —   | —   | D D D                            | D D D  | D D S S                                     | D D D S |
| 10 <sup>-6</sup>                    | —   | —   | D D D                            | D D D  | D S S S                                     | S S S S |
| 10 <sup>-7</sup>                    | —   | —   | D D                              | D D    | D S   | S S     |

of protective antibody remained in an effective form in solution. This is true even though the serum had been absorbed with a large quantity of living pneumococci. It is apparent that if the antibody were bound *in vitro* by the Pneumococcus, it would be thrown out of solution together with the Pneumococcus by centrifugation and Berkefeld filtration. In the unextracted serum this occurred, and protective action could no longer be demonstrated. In the serum from which lipoids had been extracted, however, sufficient antibody remained in solution to protect all mice against 10<sup>-6</sup> cc. of culture, and to protect some mice against as much as 10<sup>-4</sup> cc. These results

indicate that repeated absorption with homologous pneumococci does not entirely remove the protective antibody from antipneumococcus horse serum from which the lipoids have been extracted.

*Restoration of Type Specific Agglutinating and Precipitating Properties of Extracted Type I Antipneumococcus Horse Serum*

Since it had been found that, after the removal of lipoids by alcohol and ether extraction in the cold, antipneumococcus horse serum showed no visible agglutination of homologous pneumococci or precipitation with the specific capsular polysaccharide, it became important to ascertain which of the various lipoids extracted from the serum was responsible for this loss of *in vitro* activity.

To study this point the various lipoids which had actually been removed from antipneumococcus horse serum were recovered from the alcohol and the ether supernatant liquids by vacuum distillation at low temperature. The residues obtained were suspended in an amount of saline equal to the original serum volume. These suspensions, both separately and combined, were mixed with an equal volume of extracted antipneumococcus horse serum. After incubating the mixtures for 2 hours at 37°C., they were tested to determine their ability to agglutinate homologous pneumococci, and to precipitate with the specific capsular polysaccharide. In every instance the addition of the recovered lipoids to the extracted serum failed to restore the type specific reactions *in vitro*, and no visible agglutination or precipitation could be demonstrated.

Although suspensions of the mixed lipoids recovered from the alcohol and ether supernatant liquids, following the extraction of antipneumococcus horse serum, failed to restore type specific agglutinating and precipitating properties of extracted serum, it was determined to study the effects of a series of separate purified lipid substances upon the specific reactions of the extracted serum.

To this end, lecithin was prepared from egg yolk, by the method of Levene and Rolf (14), and cephalin was prepared from calf brain by the method of Page and Bulow (15). These two phospholipins were suspended in saline at the desired dilutions. The cholesterol used was a commercial preparation of orthocholesterin (Schuchardt), which was dissolved in a small quantity of alcohol, and suspensions in saline at desired dilutions were made from this solution. Equal parts of dilutions of extracted horse serum, lipid suspension and phosphate buffer were mixed, incubated for 2 hours at 37°C. and kept in the ice chest overnight. An amount of a suspension of heat-killed Type I pneumococci equal to

the volume of the mixture was then added. Readings were made after 2 hours at 37°C. and overnight in the ice chest. The results of agglutination tests with the mixtures of extracted immune horse serum and various lipid suspensions are shown in Table VI.

Lecithin in very small amounts restores the type specific agglutinating property of extracted Type I antipneumococcus horse serum. This reactivation of the extracted serum is most readily demonstrated when the lipid suspension and the extracted serum are buffered at pH 6.0. Cephalin, on the other hand, is without effect, and when

TABLE VI

*Restoration of Agglutinating Property of Extracted Type I Antipneumococcus Horse Serum*

| Type I antipneumococcus horse serum      | Lipoid added         | Suspension of heated killed Type I pneumococci |      |
|--|----------------------|--|------|
|  |                      | pH of reacting mixture                         |      |
|  |                      | 6.0  | 7.0  |
| Before extraction.....                   | —                    | ++++   | ++++ |
| After extraction (Method A).....         | —                    | —  | —    |
| “ “ “ “ .....                            | Lecithin 1:10,000    | ++++   | ++   |
| “ “ “ “ .....                            | Cephalin 1:10,000    | —  | —    |
| “ “ “ “ .....                            | Cholesterol 1:10,000 | —  | —    |
| “ “ “ “ .....                            | Cholesterol 1:40,000 | —  | —    |
|  | followed by          |  |      |
|  | Lecithin 1:10,000    | —  | —    |
| No serum.....                            | Lecithin 1:10,000    | —  | —    |
| Type II antipneumococcus horse serum ... | Lecithin 1:10,000    | —  | —    |

All sera were tested at a final dilution of 1:20.

mixed with extracted horse serum fails entirely to restore the property of type specific agglutination. The addition of cholesterol not only fails to restore the agglutinating property to extracted horse serum but exerts an inhibitory action upon the reactivation by lecithin. When minute amounts of cholesterol are mixed with the extracted horse serum prior to the addition of lecithin, the restoration of the type specific agglutinating property is prevented.

From these results it is evident that the restoration of the specific agglutinating property of extracted antipneumococcus horse serum

occurs only in the presence of one particular phospholipin, lecithin. The fact that cholesterol inhibits lecithin reactivation may explain the previous finding that the mixed lipoids recovered from the extracting solvents failed to restore *in vitro* activity to extracted horse serum.

*Properties of Type I Antipneumococcus Rabbit Serum after Extraction of Lipoids*

Type I antipneumococcus rabbit serum from which lipoids had been extracted by means of alcohol and ether (Method A), and by alcohol, petroleum ether and ether (Method B), a somewhat more drastic procedure than Method A, was

TABLE VII

*Comparison of Agglutination and Precipitation Reactions of Type I Antipneumococcus Rabbit Serum, before and after Extraction of Lipoids by Method A and Method B*

|   | Serum dilution          | Type I antipneumococcus rabbit serum |  |  |
|---|-------------------------|--------------------------------------|--|--|
|   |                         | Before extraction<br>Lot 7           | After extraction<br>(Method A)<br>Lot 7 (A.E.) | After extraction<br>(Method B)<br>Lot 7 (A.P.E.) |
| Agglutination of Type I Pneumococcus              | 1:10                    | ++++                                 | +++  | ++±  |
|   | 1:20                    | ++++                                 | ++   | ±  |
|   | 1:40                    | ++                                   | ++   | —  |
|   | 1:80                    | +                                    | ±  | —  |
|   | 1:160                   | —                                    | —  | —  |
|   | Polysaccharide dilution |                                      |  |  |
| Precipitation with Type I capsular polysaccharide | 1:10,000                | ++                                   | ++   | ±  |
|   | 1:40,000                | +++                                  | +++  | —  |
|   | 1:400,000               | +++                                  | ++±  | —  |
|   | 1:4,000,000             | ++                                   | ±±   | —  |

studied to determine what effect the removal of lipoids might have upon the various immunological characteristics of rabbit serum. The results of agglutination tests with homologous pneumococci, and precipitation tests with the specific capsular polysaccharide, are shown in Table VII.

Contrary to the changes found in extracted immune horse serum, no significant differences were demonstrable in the immunological characteristics of immune rabbit serum from which lipoids had been extracted by alcohol and ether (Method A). After this extraction, immune rabbit serum agglutinated homologous pneumococci and precipitated with the capsular polysaccharide in undiminished titer,

protected mice against fatal infection with Type I pneumococci without exhibiting any prozone, and fixed complement with the type specific carbohydrate just as did untreated rabbit serum. After the extraction of lipoids from Type I antipneumococcus rabbit serum by Method B, however, there is a striking reduction in the titer at which the serum agglutinates homologous pneumococci. Although the agglutinating property is not entirely lost, as it appears to be with extracted immune horse serum, there is a definite quantitative reduction in titer after the removal of lipoids from immune rabbit serum. The precipitating capacity of extracted rabbit serum is so markedly reduced that only a faint cloudiness occurs in the presence of type specific polysaccharide in a concentration as great as 1:10,000.

TABLE VIII

*Protection of Mice against Fatal Infection with Type I Pneumococci, by Extracted Type I Antipneumococcus Rabbit Serum*

| Type I culture   | Type I antipneumococcus rabbit serum 0.05 cc. |   | Virulence control |
|------------------|---|---|-------------------|
|                  | Before extraction<br>Lot 15                   | After extraction<br>(Method B)<br>Lot 15 (A.P.E.) |                   |
| cc.              |   |   |                   |
| 10 <sup>-1</sup> | D 24  | D 24  | —                 |
| 10 <sup>-2</sup> | S   | S   | —                 |
| 10 <sup>-3</sup> | S   | S   | —                 |
| 10 <sup>-4</sup> | S   | S   | —                 |
| 10 <sup>-5</sup> | —   | —   | —                 |
| 10 <sup>-6</sup> | —   | —   | D 48              |
| 10 <sup>-7</sup> | —   | —   | D 48              |
| 10 <sup>-8</sup> | —   | —   | D 48              |

These results are in contrast to the loss of visible type specific agglutination and precipitation by antipneumococcus horse serum following the extraction of lipoids. It may be stated that, with the methods used, it has not been possible to remove sufficient lipid from antipneumococcus rabbit serum to cause complete loss of these specific *in vitro* reactions. However, the significant reduction in type specific agglutination and precipitation following extraction seems to indicate that lipoids exert no inconsiderable rôle in the manifestations of *in vitro* activity of antipneumococcus rabbit serum.

The results of protection tests with extracted immune rabbit serum are shown in Table VIII.

As in the case of extracted horse serum, the extraction of lipoids

in no way altered the protective action of immune rabbit serum. Both before and after extraction, 0.05 cc. of antipneumococcus rabbit serum protected mice against  $10^{-2}$  cc. of Type I pneumococcus culture.

In order to determine whether or not the extraction of lipoids from antipneumococcus rabbit serum had any effect upon complement fixing power, the extracted serum was tested with homologous pneumococci, and also with capsular polysaccharide. It was found that the extracted serum bound complement as readily as the unextracted rabbit serum. The extraction of lipoids from rabbit serum by alcohol, petroleum ether and ether (Method B) therefore does not alter this specific characteristic of immune rabbit serum.

*Restoration of Specific Agglutinating and Precipitating Properties of  
Type I Antipneumococcus Rabbit Serum after  
Extraction of Lipoids*

Since it had been shown that the type specific agglutinating and precipitating properties of antipneumococcus horse serum from which lipoids had been removed can be restored by the addition of lecithin, it was of some importance to study the effect upon extracted rabbit serum of the addition of separate lipid substances. Although the removal of lipoids from antipneumococcus rabbit serum did not result in a complete loss of *in vitro* activity, as determined by the agglutination and precipitation tests, the reduction in titer was sufficient to permit an evaluation of the effectiveness of various lipoids in restoring the full *in vitro* activity to the extracted serum.

Suspensions of lecithin, cephalin and cholesterol, in the same concentrations used in the study of extracted horse serum, were mixed separately with equal parts of diluted immune rabbit serum extracted by Method B. The pH of the mixtures was adjusted by the addition of phosphate buffer. These preparations were incubated for 2 hours at 37°C. and kept in the ice chest overnight. Agglutination tests with the lipid-serum mixtures were carried out in a manner identical to that used with extracted horse serum. The results are presented in Table IX.

Cephalin in very minute amounts restores to its original value the markedly reduced type specific agglutinating power of extracted Type

I antipneumococcus rabbit serum. This effect is most readily demonstrable when the lipid suspension and extracted serum are buffered at pH 7.0. Lecithin, however, is entirely without effect, and does not restore the specific agglutinating activity of extracted rabbit serum. As was found in the study of extracted horse serum, cholesterol does not restore the agglutinating power of rabbit serum from which lipoids have been extracted, and in its presence reactivation by cephalin is inhibited.

TABLE IX

*Restoration of Agglutinating Property of Extracted Type I Antipneumococcus Rabbit Serum*

| Type I antipneumococcus horse serum | Lipoid added         | Suspension of heat-killed Type I pneumococci |      |
|-------------------------------------|----------------------|--|------|
|                                     |                      | pH of reacting mixture                       |      |
|                                     |                      | 6.0  | 7.0  |
| Before extraction.....              | —                    | ++++   | ++++ |
| After extraction (Method B).....    | —                    | ±  | ±    |
| “ “ “ “ .....                       | Cephalin 1:10,000    | ++   | +++  |
| “ “ “ “ .....                       | Lecithin 1:10,000    | ±  | ±    |
| “ “ “ “ .....                       | Cholesterol 1:10,000 | —  | —    |
| “ “ “ “ .....                       | Cholesterol 1:40,000 |  |      |
|                                     | followed by          | ±  | ±    |
|                                     | Cephalin 1:10,000    |  |      |
| No serum.....                       | Cephalin 1:10,000    | —  | —    |

The sera were tested at a final dilution of 1:20.

These results indicate that the type specific agglutinin and precipitin titers of antipneumococcus rabbit serum are markedly reduced by the extraction of lipoids (Method B), and, further, that it is possible to restore full activity by the addition of one particular phospholipin, that is, cephalin.

## COMMENT

It has been shown that the lipoids of both Type I antipneumococcus horse serum and Type I antipneumococcus rabbit serum are essential to the demonstration of certain specific immunological reactions *in vitro*. After the extraction of lipoids there was no visible evidence of specific precipitation or agglutination by antipneumococcus horse serum, and these properties of antipneumococcus rabbit serum were

greatly reduced. In both instances it has been demonstrated that the addition of one separate phospholipin to the extracted sera restored the initial type specific *in vitro* activity. In the case of extracted immune horse serum, lecithin selectively restored type specific agglutination and precipitation, while extracted immune rabbit serum regained its type specific agglutinating and precipitating properties after the addition of cephalin.

The protective antibody of extracted horse serum was only partially absorbed by washed pneumococci in contradistinction to the complete absorption of the protective antibody from unextracted horse serum.

Of the numerous characteristics which distinguish Type I antipneumococcus horse serum from Type I antipneumococcus rabbit serum, only the water solubility of the immune globulin has been shown definitely to depend upon the presence of lipoids. The water solubility of the immune globulin fraction of extracted horse serum very closely resembles that of the globulin fraction of immune rabbit serum.

In considering the significance of these results, it is of importance to distinguish somewhat sharply between the two stages of the reaction between antigen and antibody. The first stage of the reaction, that is, the combination between antigen and antibody, is not as markedly affected by an increase or decrease in non-specific substances as is the second stage. The second stage of the reaction between antigen and antibody is the result, probably, of a series of complex processes, in which a number of non-specific factors are known to be of prime importance. For example, the processes of agglutination and precipitation are markedly influenced by salt concentration. In salt-free mixtures these secondary and visible phenomena do not occur (Northrop and De Kruif (16), and Eagle (17)). Likewise, high concentrations of salt inhibit precipitin and agglutinin reactions (Landsteiner and Welecki (18)). The effect of hydrogen ion concentration is also well known, although in most agglutination and precipitation reactions this can be varied through rather wide limits without strikingly changing the results. The effects of the addition of non-specific proteins to immune serum are to some extent dependent upon the concentration of antibody, and may either increase or decrease the sensitivity of the reaction (Sobotka and Friedländer (19)). Various non-specific organic substances such as glucose (Downs and Good-



ner (20)), and cane sugar (Landsteiner and Welecki (18)), can considerably reduce or completely inhibit these secondary reactions.

The loss of type specific agglutination and precipitation after the extraction of lipoids from antipneumococcus horse serum may be explained simply as the result of secondary non-specific effects comparable to the known changes induced by other non-specific agents. That is, it is conceivable that the removal of lipoids sufficiently shifts the physicochemical equilibrium of the reactive system so as to prevent the secondary changes of visible flocculation and precipitation from taking place after the primary union of antigen and antibody has occurred.

If this explanation should prove to be correct, it should eventually be possible to demonstrate that despite the absence of the secondary phenomena, the primary reaction, that is, combination between antigen and antibody, actually occurs. It appears from the results of absorption tests that only a fraction of the total type specific antibody of extracted horse serum was removed by absorption with pneumococci, whereas with unextracted serum all the protective antibody was absorbed. This may be taken to indicate that some, at least, of the antibody of extracted serum did enter into combination with the bacterial cells. As yet, however, it has not been possible to reach a conclusive decision as to whether or not union of antigen and antibody actually occurs in an immune serum from which lipid has been extracted.

If, on the other hand, no combination actually occurs between pneumococcus and antibody, after the extraction of lipid from immune serum, it becomes necessary to assume that the antibody is a complex lipoprotein, and not simply modified pseudoglobulin. The effect of the addition of certain individual lipoids to extracted serum, and the resulting restoration of the secondary phenomena of agglutination and precipitation by one single phospholipin, the nature of which is predicated by the species from which the serum is derived, suggests that the antibody may be a phospholipin-globulin complex. Under these conditions it is apparent that neither the globulin nor the phospholipin alone would carry the complete properties of the whole antibody. It would be possible to demonstrate the characteristic and type-specific properties of the whole antibody only when the necessary phospholipin and globulin were associated.

It is apparent that in the case of antipneumococcus horse serum, at least, the visible phenomena of the second phase of the agglutinin and precipitin reactions do not occur in the absence of lipoid. Whether this is a non-specific effect, somewhat analogous to the failure of agglutination and precipitation in the absence of salt, or a specific result due to the removal of an essential constituent of the antibody complex, can be determined only by further experimentation.

### CONCLUSIONS

It has been demonstrated that the removal of lipoids from Type I antipneumococcus horse serum causes a loss of the visible phenomena of type specific agglutination and precipitation, and in the case of rabbit serum a marked reduction in these properties. Initial activity of the type specific antibody can be restored to extracted immune horse serum by the addition of lecithin, and to rabbit serum by the addition of cephalin. The significance of these observations in respect to the relation of phospholipins to the type specific reactions of antipneumococcus serum is discussed.

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# IMMUNOLOGICAL STUDIES WITH THE VIRUS OF INFLUENZA\*

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On the basis of clinical observations, a widespread opinion prevails that immunity to influenza is of brief duration. This has usually been explained as due to one of two circumstances: (1) An attack of influenza is not followed by the formation of specific antibodies in the serum of the patient, or, if so, they persist for only a short time. (2) Many strains of the infectious agent are commonly in circulation, and the human individual may be attacked repeatedly by strains of different serological specificity. The lack of knowledge of the nature of the causative agent, and the failure to transmit the disease consistently to laboratory animals, have made it impossible heretofore to submit the various hypotheses to experimental tests.

Recent studies (1-3), however, appear to have established the fact that the primary causative agent of human influenza is a filterable virus. In this laboratory the virus has been recovered from the nasopharynx of patients suffering from influenza in Puerto Rico and Philadelphia. These strains, (designated P.R.8 and Phila.) have been repeatedly transferred both in ferrets and mice. In both species of animals an experimental disease characterized by involvement of the respiratory tract, especially of the lungs, has been produced. Serum of ferrets recovered from infection with either of these strains of virus was found to neutralize the infectivity of both strains for mice. Furthermore, the serum of a horse immunized by Andrewes, Laidlaw and Smith (2) against a strain of virus (W.S.) recovered in England, was also found to neutralize both the P.R.8 and Phila. strains of virus. The serum of swine and ferrets which neutralized the virus

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of swine influenza (Shope (4)) failed, however, to neutralize the recently isolated human strains of virus. These results (5) show that the strains of virus recovered from human cases of influenza are immunologically identical, while the virus of swine influenza differs from them serologically. Nevertheless, certain evidence to be presented in the present paper indicates that an immunological relationship exists between the strains of human and swine influenza virus. Similar observations have been reported by the English investigators (6) who have, in addition, recovered several immunologically identical strains of human virus from different outbreaks of influenza in England.

The present report deals with additional studies of the immunity reactions of animals to the P.R.8 and Phila. strains of virus, and with certain results obtained with human sera which indicate the etiological relationship of the virus to the human disease.

### *Materials and Methods*

*Strains of Virus.*—The strains of virus used in the present study were obtained from sputum or pharyngeal washings of patients suffering from influenza in Puerto Rico (P.R.8) and Philadelphia. In ferrets the disease produced by the P.R.8 strain is somewhat more severe than that produced by the Phila. strain. The pulmonary lesions in ferrets infected with the P.R.8 strain are usually more extensive, and the respiratory symptoms are more marked. The characteristics of the experimental disease produced by these two strains in mice are identical.

*Mice.*—Albino mice of the Swiss strain were used throughout. Young mice 4–6 weeks of age are most satisfactory. In older mice irregular survivals are more frequent, and death of the infected animals is somewhat delayed.

*Neutralization Tests.*—The technique of the virus neutralization tests has previously been described (5). The serum to be tested is mixed with a 10 per cent suspension of infected mouse lung in saline, and, after incubation, 0.03 cc. of the mixture is inoculated into the nasal passages of 4 or 5 anesthetized mice. If the mice die, their lungs are removed and the extent of the pulmonary consolidation is noted. All surviving mice are killed on the 6th day after infection, and the extent of pulmonary involvement recorded. The absence of pulmonary lesions in mice receiving virus and serum is taken to indicate the fact that the serum possesses specific neutralizing antibodies.

*Isolation.*—When ferrets are received from the breeders they are placed in quarantine for a period of 2–4 weeks and observed for any signs of illness. During the winter certain of the animals were found to carry hemolytic streptococci or small Gram-negative bacilli in the respiratory tract, and among these animals

purulent rhinitis sometimes occurred. Such animals were segregated from other stock.

After this period of observation, the ferrets are transferred to another quarantine room, from which they are transported to the isolation units when needed for experimental purposes. At no time are they exposed to experimentally infected animals or to persons who have contact with infected animals.

### *Active Immunity in Ferrets*

In a previous publication (5) it was shown that the serum of ferrets which have recovered from infection with either the P.R.8 or Phila. strain of influenza virus is capable of passively protecting white mice against both strains of virus.

In addition to possessing neutralizing antibodies in their blood, ferrets recovering from infection with one strain have been found to be actively immune to reinfection with either the homologous or heterologous strain. The duration of this active immunity is somewhat variable. In most instances, tests made 4 months after previous infection have shown the animals to be resistant to reinfection. In certain instances, however, reinoculation has been followed by a single sharp rise of temperature in the first 24 hours, without subsequent evidence of infection. Reactions of this type have been considered to be either non-specific or of the nature of accelerated immune reactions.

These results appear to indicate clearly that the active immunity which follows infection with one strain is fully effective against reinfection with the other strain. The fact that a comparatively firm active immunity may persist for months in previously infected ferrets is in complete agreement with the observations of the British workers (1, 6). Infrequently, however, reinfection has occurred in animals receiving a second intranasal inoculation within a period of 2 months. In these cases the only evidence of reinfection was the persistence of fever for 2-3 days after the second inoculation.

It was of interest, therefore, to note that one shipment of animals from a known susceptible stock was found to be resistant to experimental infection. Another group of animals from the same source was completely susceptible. A third group, obtained from this breeder, on arrival at the laboratory exhibited nasal discharge. Certain individual animals of this latter group, when used for experi-

mental purposes and inoculated intranasally with active influenza virus, developed no febrile or constitutional reaction, and attempts to recover virus from the nasal mucous membranes or lungs of such animals were unsuccessful. In other instances a moderate fever without other symptoms persisted for 3-4 days after inoculation, but the inoculation of suspensions of the lungs and turbinates of these animals into normal susceptible ferrets failed to produce the experimental disease. It was assumed, therefore, that these ferrets had become immune, although they had not been subjected to experimental infection.

To test this assumption, serum was obtained from 5 ferrets of the third group and of several other ferrets which had been kept in the same room for a period of months. The serum of 2 of the 5 ferrets of this group was found to contain antibodies which neutralized the P.R.8 virus in mouse tests.

6 months after they had been received from the dealer, the 2 ferrets whose serum contained neutralizing antibodies, together with a third animal of the same group which did not possess neutralizing antibodies, were inoculated simultaneously with the Phila. virus. The control animal developed fever in 24 hours, lost its appetite and exhibited catarrhal rhinitis and mild respiratory distress. When sacrificed on the 4th day after the onset of fever, involvement of practically all of both lower lobes of the lung was found. The 2 ferrets, presumably immune, developed fever on the 2nd day after inoculation, but remained well thereafter. When sacrificed on the 4th day after the development of fever, the lungs of both animals were normal in appearance. The turbinates were somewhat mucoid.

From one of these latter animals a 20 per cent suspension of ground lung and turbinate tissue was made and inoculated intranasally into a normal ferret. Likewise, the lung and turbinates of the control animal were ground to form a 10 per cent suspension and administered to another normal ferret. The animal which received the latter material became sick, and when sacrificed on the 4th day pulmonary consolidation was found. The ferret which received the passage material from the animal possessing neutralizing antibodies had a mild febrile reaction on the 2nd day, but appeared perfectly well. When sacrificed on the 4th day, no pulmonary involvement was observed, although the nasal mucous membranes were swollen and contained some mucopurulent material from which hemolytic streptococci were recovered.

The fact that the two ferrets which possessed circulating neutralizing antibodies exhibited only a brief febrile reaction and failed to develop lung lesions, in contrast to the normal control animal of the same group, is of interest. Furthermore, passage from one of these 2 animals failed to elicit more than a minimal febrile reaction in a

normal ferret, whereas the animal which received material from the control ferret developed distinct pulmonary lesions. This indicates that following the experimental infection of the ferrets whose serum contained neutralizing antibodies the virus was either completely neutralized or persisted in only small amounts, since heavy suspensions of the tissues of these animals failed to induce a typical infection when injected into a normal ferret.

The fact that immune ferrets may be encountered among stock animals not infected experimentally must be taken into account in all studies relating to immunity. Whether the neutralizing antibodies present in the serum of these animals develop solely in response to contact infection of human origin, is a problem the solution of which must await further study. In view of these observations, it is extremely important that strict isolation be carried out in the care of animals used for experimental purposes.

### *Immunization of Rabbits*

During the course of experiments with Rift Valley fever virus, it was found that rabbits which were inoculated intraperitoneally with the virus presented no evidence of infection. These animals, however, subsequently developed in the circulating blood neutralizing antibodies for the virus of Rift Valley fever (7).

It seemed important, therefore, to determine whether rabbits inoculated intraperitoneally with the influenza virus subsequently developed demonstrable neutralizing antibodies in their serum.

Two adult male Chinchilla rabbits were used. Serum was obtained for control purposes before inoculation. Both animals were then given, intraperitoneally, 5.0 cc. of a 10 per cent emulsion of lung and turbinates of a ferret infected with the P.R.8 strain of influenza virus. No fever or other evidence of infection occurred. 28 days later a similar inoculation was made by the same route. The serum obtained 14 days after the second injection was found to contain antibodies which neutralized both the P.R.8 and Phila. strains of virus in mice, while the serum before inoculation was ineffective. This immune serum was tested by Shope against the virus of swine influenza and found to neutralize that virus in mouse tests. Similar results have been obtained in rabbits inoculated with the Phila.



strain of virus. This evidence, like that obtained in the case of Rift Valley fever, indicates that rabbits, although apparently insusceptible to infection, are nevertheless capable of giving rise to the formation of specific antibodies in response to the antigenic action of the virus.

### *Immunization of Mice*

The virulence of influenza virus for mice has been progressively enhanced by repeated passage through these animals. The virulence of the strains used in the present studies is such that 0.03 cc. of a 1:1000 dilution of infected mouse lung inoculated intranasally produces a fatal infection, and a similar amount of a 1:10,000 dilution produces pulmonary lesions from which the mice may recover. In contrast to the virulence by the intranasal route, it has been repeatedly observed that as much as 0.2 cc. of a 10 per cent suspension subcutaneously, or 0.5 cc. intraperitoneally, produces no evidence of infection. Similarly, suspensions of infected ferret lung or Berkefeld filtrates of lungs and turbinates which contain active virus, as shown by intranasal infection, produce no evidence of infection in mice when injected in large amounts by the subcutaneous or the intraperitoneal route. It was found, however, that animals so treated become actively resistant to intranasal infection with the virus.

*Immunization of Mice with Influenza Virus (Strain P.R.8).*—Each of 20 normal young mice was given, subcutaneously, 0.2 cc. of a 10 per cent broth emulsion of the lungs of mice infected with P.R.8 strain of human influenza virus. As controls, 20 normal mice were each given 0.2 cc. of a 10 per cent suspension of normal mouse lung by the same route. After 14 days the virus-vaccinated mice each received, intraperitoneally, 0.2 cc. of a 10 per cent suspension of the lungs of mice infected with P.R.8 virus. At the same time, the control mice each received the same amount, intraperitoneally, of a 10 per cent suspension of normal mouse lung. 1 week after the second injection, all mice, slightly anesthetized with ether, were inoculated intranasally with 0.05 cc. of a 10 per cent suspension of P.R.8 mouse virus. By the 6th day after inoculation all but one of the control mice vaccinated with normal mouse lung were dead, and at autopsy all exhibited consolidation of both lungs. The virus-vaccinated mice were all well and presented no evidence of infection. 6 of these mice were sacrificed and their lungs examined. No pulmonary lesions were detected. The remaining 14 mice which had been found actively immune to intranasal infection with the strain of virus used for vaccination were then retested to determine their resistance to a strain other than that used for immunization. To this end they were inoculated intra-

nasally with 0.05 cc. of a 10 per cent suspension of the Phila. strain of virus. All the vaccinated mice were found to be immune to the second strain of virus.

Similar results have been obtained in mice vaccinated against the Phila. strain of virus and subsequently retested with the P.R.8 strain of mouse virus. Furthermore, the serum of mice so treated has been found to contain antibodies which neutralize the infectivity of both strains of virus. In a similar manner mice have been vaccinated with virus-containing material derived from infected ferrets. Both unfiltered suspensions of virus material and Berkefeld filtrates of these suspensions have been used. The resultant active immunity appears to be quite as effective as that obtained in mice vaccinated with mouse passage virus. The living virus, therefore, functions as an immunizing agent irrespective of the species of animal from which the infectious material is derived. Smith, Andrewes and Laidlaw (8), in a recent article, have described their results regarding the immunization of mice by the subcutaneous inoculation of mouse virus.

Different intervals between the vaccinating injections have been tried, and the interval between the last vaccinating dose and the intranasal infection has been varied. The best results have been obtained, so far, by giving 3 consecutive subcutaneous injections at 7-10 day intervals, or with a single subcutaneous dose followed 2 weeks later by an intraperitoneal injection. Single subcutaneous or intraperitoneal doses of living virus have not, up to the present, resulted in effective immunity. Nevertheless, mice recovering from the experimental disease induced by intranasal infection have been found actively resistant to reinfection.

Certain groups of these immunized mice have been tested by Shope and found to be actively resistant to infection with swine influenza virus as well. The significance of these results will be considered in a later publication.

#### *Neutralization Tests with Serum of Patients Suffering from Respiratory Infections*

Serum was obtained from 3 patients admitted to the Hospital of The Rockefeller Institute suffering from influenza. The serum of these individuals during the acute and convalescent stages of the

disease was tested for the presence of neutralizing antibodies against both the P.R.8 and Phila. strains of influenza virus. In comparison with normal ferret or normal horse serum, most human sera have an inhibitory effect upon the activity of the virus. Nevertheless, the serum of these individuals taken during the acute stage of influenza failed to prevent the development of pulmonary lesions in mice inoculated with serum-virus mixtures, while the convalescent sera uniformly protected the animals. Furthermore, the antibodies develop-

TABLE I

*Neutralization Tests in Mice with Serum of Influenza Patients*

| Serum |                   | Influenza virus (P.R.8 strain)        |       |       |       |
|-------|-------------------|---------------------------------------|-------|-------|-------|
|       |                   | Severity of pulmonary lesions in mice |       |       |       |
|       |                   | No. 1                                 | No. 2 | No. 3 | No. 4 |
| H.F.  | Acute.....        | +++++                                 | +++++ | +++   | +++   |
|       | Convalescent..... | 0                                     | 0     | 0     | 0     |
|       | 6 mos. later..... | 0                                     | 0     | 0     | 0     |
| B.P.  | Acute.....        | ++                                    | ++    | +++   | ++    |
|       | Convalescent..... | 0                                     | 0     | 0     | ±     |
|       | 6 mos. later..... | 0                                     | 0     | 0     | ±     |
| M.B.  | Acute.....        | ++                                    | +     | ++    | +++   |
|       | Convalescent..... | 0                                     | 0     | 0     | 0     |
|       | 6 mos. later..... | 0                                     | 0     | 0     | 0     |

0 = no pulmonary lesion.

± to +++++ = progressive degrees of pulmonary involvement.

ing in early convalescence were found to persist for 6-8 months at least.

Similar tests were made with the serum of patients acutely ill with, and convalescent from, pneumococcus pneumonia. In general, the effect of convalescent pneumonia serum upon the influenza virus appeared to be no different from that of the serum taken at the height of the illness. Neutralizing antibodies for the influenza virus did not develop in response to the pneumococcus infection. If neutralizing antibodies were present at the onset of the illness, they were likewise present in the convalescent serum. In the one instance in which

neutralizing antibodies did develop in convalescence from pneumonia, it seemed quite likely from the clinical history that influenza may have been associated with the onset of the pneumonia.

TABLE II  
*Neutralization Tests in Mice with Serum of Pneumonia Patients*

| Serum                        |                   | Influenza virus (P.R.8 strain)        |       |       |       |
|------------------------------|-------------------|---------------------------------------|-------|-------|-------|
|                              |                   | Severity of pulmonary lesions in mice |       |       |       |
|                              |                   | No. 1                                 | No. 2 | No. 3 | No. 4 |
| M.C.                         | Acute.....        | 0                                     | 0     | 0     | 0     |
|                              | Convalescent..... | 0                                     | 0     | 0     | 0     |
| J.C.                         | Acute.....        | +++                                   | ++    | ++    | 0     |
|                              | Convalescent..... | +++                                   | ++    | +     | 0     |
| A.W.                         | Acute.....        | ++                                    | ±     | +     | ±     |
|                              | Convalescent..... | ++                                    | +     | ++    | 0     |
| R.O.                         | Acute.....        | 0                                     | +     | 0     | ++    |
|                              | Convalescent..... | 0                                     | +     | 0     | ++    |
| C.P.                         | Acute.....        | +                                     | 0     | +++   | +     |
|                              | Convalescent..... | ++                                    | +     | +     | 0     |
| C.M.                         | Acute.....        | 0                                     | 0     | +     | 0     |
|                              | Convalescent..... | 0                                     | 0     | 0     | +     |
| H.C.                         | Acute.....        | +++                                   | +++   | ++    | +++   |
|                              | Convalescent..... | +++                                   | +++   | ++    | ++++  |
| B.W.                         | Acute.....        | +                                     | ++    | ++    | +     |
|                              | Convalescent..... | 0                                     | 0     | 0     | 0     |
| Control<br>influenza<br>H.F. | Acute.....        | +++                                   | ++    | ++    | +++   |
|                              | Convalescent..... | 0                                     | 0     | 0     | 0     |

0 = no pulmonary lesions.

± to ++++ = progressive degrees of pulmonary involvement.

Further studies were made with the serum of 4 individuals taken before, during and after the course of a common cold. In the 4 cases studied, the effect of the individual's serum on the influenza virus was entirely uninfluenced by the common cold.

These results indicate that the development of antibodies which neutralize the influenza virus is a specific response to the infectious agent, and that the virus is causally related to the human disease—*influenza*.

TABLE III

*Neutralization Tests in Mice with Serum of Human Individuals before, during and after a Common Cold*

| Serum                 | Influenza virus (P.R.8 strain)        |       |       |       |
|-----------------------|---------------------------------------|-------|-------|-------|
|                       | Severity of pulmonary lesions in mice |       |       |       |
|                       | No. 1                                 | No. 2 | No. 3 | No. 4 |
| R.L. Before cold..... | ++                                    | +     | ++    | ++    |
| During " .....        | +                                     | +     | ++    | +     |
| After " .....         | ++                                    | +     | ++    | +     |
| T.A. Before cold..... | +                                     | +     | ++    | ++    |
| During " .....        | +                                     | ++    | +     | +     |
| After " .....         | ±                                     | ++    | ±     | +     |
| F.H. Before cold..... | 0                                     | ±     | 0     | 0     |
| During " .....        | 0                                     | 0     | 0     | 0     |
| After " .....         | 0                                     | +     | 0     | 0     |
| T.F. Before cold..... | +++++                                 | +++   | +++   | +     |
| During " .....        | +++++                                 | +++++ | +++++ | +++++ |
| After " .....         | ++                                    | +++++ | ++    | ++    |

0 = no pulmonary lesions.

± to +++++ = progressive degree of pulmonary involvement.

#### DISCUSSION

The evidence presented in this report demonstrates that a state of immunity as measured by circulating antibodies and active resistance follows recovery from infection with the virus of influenza. That the mere presence of neutralizing antibodies in the circulating blood may not necessarily assure a complete refractory state to reinfection is recognized. Ferrets which have developed neutralizing antibodies following experimental or presumably direct infection exhibit little or no reaction to reinfection. In certain instances, however, reinoculation may elicit a brief febrile reaction without other evidence of in-

fection. Attempts to recover virus from these animals have in general been unsuccessful. The results in experimental animals indicate that although the immunity acquired as a result of infection may not be sufficiently absolute to prevent febrile reactions on reinfection, the virus is quickly neutralized and from these animals is not so readily recoverable as it is from normal animals infected for the first time. If a similar set of circumstances prevails in the natural disease in man, the experimental results suggest a possible explanation for the lack of uniform success in attempts to recover virus from all patients with influenza.

Virus neutralization tests with serum of influenza patients taken during the acute stage of the disease, during early convalescence and at later periods, have shown that the serum of the individual at the height of the disease fails to neutralize the influenza virus, whereas serum taken from the same patient during convalescence does contain specific antibodies. These antibodies are not evanescent, but persist for several months at least, as evidenced by the neutralizing capacity of serum obtained from patients 6-8 months after recovery from influenza.

Similar studies with the serum of patients ill with, and recovering from, pneumococcus pneumonia, have shown that in general specific antibodies neutralizing the influenza virus do not develop in response to pneumococcus infection. Studies of the antibody content of the serum of human individuals before, during and after a common cold were made. The results indicate that this type of respiratory infection does not stimulate the formation of antibodies against the virus of influenza. It appears, therefore, that the neutralizing action of the serum of human individuals is a specific response to infection with the influenza virus.

That the virus of swine influenza is not serologically identical with the strains of virus recently isolated from human cases of influenza seems definitely established (5). Nevertheless, that the strains of human influenza virus and of swine influenza virus are related is shown by the active cross-immunity in ferrets (6), and in mice immunized with the P. R. 8 or Phila. virus, as well as by passive neutralization of swine influenza virus by the serum of rabbits immunized with P. R. 8 virus. Further consideration of this problem will form the basis of a

subsequent report. It has been suggested, moreover, that the virus of swine influenza is the etiological agent which gave rise to the 1918 pandemic of influenza in man (6). If this is subsequently shown to be true, the problem of immunity to influenza will of necessity involve consideration of the possible existence of multiple strains of virus of related but not wholly identical antigenic structure.

#### SUMMARY

Following infection with the virus of influenza, both ferrets and mice develop a state of active immunity to reinfection. The serum of these animals contains neutralizing antibodies, as evidenced by the capacity of the serum to confer passive protection to mice against infection with the P.R.8 and Phila. strains of the virus of human influenza.

Rabbits which are apparently insusceptible to infection with the virus of influenza produce specific antibodies in response to repeated injection of virus-containing material. The serum of immunized rabbits affords passive protection to mice against mouse-virulent virus.

Although the subcutaneous or intraperitoneal injection of the living virus does not produce infection in mice, animals so treated acquire active immunity against subsequent infection by the intranasal route.

Neutralization tests with the serum of patients before and after recovery from influenza, pneumonia and the common cold indicate that neutralizing antibodies arise as a specific response to infection with the virus of influenza.

The immunological identity of strains of influenza virus recovered from human sources has been established, and the possible existence of strains of related, but not identical, antigenic structure is discussed.

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# INACTIVATION OF POLIOMYELITIS VIRUS IN VITRO BY CRYSTALLINE VITAMIN C (ASCORBIC ACID)\*

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Inactivation of the virus of poliomyelitis by various chemicals has formed the subject of investigation for many authors. The sum total of this work has demonstrated that this virus is peculiarly susceptible to the action of certain oxidizing agents (hydrogen peroxide, potassium permanganate, etc.), while at the same time exhibiting marked resistance against such general protoplasmic poisons as phenol.

Valuable as such studies are in that they aid in an understanding of the nature of the etiological agent, they are obviously of but little significance for the problem as to what chemical forces, if any, are engaged in the inactivation of the virus in the body of the normally insusceptible host or the recovered individual. An attempt to approach this question in a rational manner was made by studying the distribution of physiological poliocidal substances throughout the body. This led to the discovery that virus-neutralizing substances were present, not only in serum but in human tears (1), placenta (2) and pregnancy urine (3), as well as in adrenal extracts containing either the medullary (adrenalin) or the cortical (cortin) hormone (4). In the course of this work it was observed that the same endocrine secretions (adrenalin, cortin) (4) as well as certain antitoxic sera (5) were frequently capable of inactivating not only poliomyelitis virus but also diphtheria toxin. When it was furthermore found that an important constituent of the adrenal gland, *i.e.* vitamin C or ascorbic acid, possessed the power of inactivating diphtheria toxin *in vitro* and *in vivo* in extraordinarily small amounts (6), it became an im-

\* This work was carried out under a grant from the Rockefeller Foundation.



portant problem to determine whether or not poliomyelitis virus is vulnerable, in a like manner, to the injurious effect of this substance.

This paper presents experiments bearing on this point.

#### EXPERIMENTAL

A total of 30 *rhesus* monkeys were injected intracerebrally with mixtures of a constant dose of virus (Aycock passage strain), *i.e.* 0.1 cc. of the supernatant of a centrifuged 10 per cent poliomyelitis cord suspension, and graded doses of vitamin C.<sup>1</sup> In addition, 5 monkeys received the same amount of virus mixed with saline or distilled water, for control purposes. The doses of vitamin C varied from as much as 100 mg. to as little as 0.05 mg. These quantities were obtained by progressive dilutions with distilled water of a freshly prepared 5 per cent solution of vitamin C, the respective doses being always contained in a volume of 1 cc.

In order to guard against a possible inactivation of the virus by the strong acid reaction of concentrated vitamin C solutions, the mother solution was adjusted at pH 6.6 to 6.8 by the addition of  $\frac{N}{10}$  sodium hydroxide, immediately before making the dilutions for the various test doses. According to Amoss (7) the virus remains viable at least 48 hours at hydrogen ion concentrations ranging from pH 5.0 to 8.2. In Howitt's (8) experiments it was not destroyed by acidification to pH 4.4. The virus-vitamin C mixtures were incubated for 1½ hours at 37°C., placed in the ice box overnight and injected the following morning intracerebrally into individual monkeys. The same technique was used in preparing the control mixtures. The injected monkeys were carefully observed for 1 month and symptoms recorded. The results obtained in this experiment are given in Table I.

As can be seen from Table I, there is a definite, fairly wide range, within which inactivation of poliomyelitis virus may be obtained by vitamin C. Absolute regularity of the phenomenon, however, is evidently limited to a narrower zone. In our experiments, the optimal quantities seem to lie between 10 and 5 mg., larger and smaller doses failing to protect with the same consistency. With the diminution of the dose below 1 mg. a gradual loss of inactivating power is clearly indicated, no neutralization whatsoever occurring with the smallest dose tested, namely 0.05 mg. An increase of the dose above 50 mg. was evidently not feasible because of the toxicity of larger amounts.

<sup>1</sup> We are greatly indebted to Merck and Co. for placing at our disposal a generous supply of cebione, their crystalline, natural vitamin C preparation.

TABLE I  
*Inactivation of Poliomyelitis Virus in Vitro by Crystalline Vitamin C (Ascorbic Acid)*

| Monkey No. | Dose of virus | Amount of vitamin C | Result                      |
|------------|---------------|---------------------|-----------------------------|
|            | cc.           | mg.                 |                             |
| Q18        | 0.1           | 100                 | Died 24 hrs.                |
| Q19        | 0.1           | 100                 | " 24 "                      |
| O67        | 0.1           | 50                  | Complete paralysis, 11 days |
| O87        | 0.1           | 50                  | No paralysis                |
| Q77        | 0.1           | 50                  | " " "                       |
| Q82        | 0.1           | 50                  | " " (?)                     |
| O88        | 0.1           | 10                  | No paralysis                |
| Q20        | 0.1           | 10                  | " "                         |
| Q75        | 0.1           | 10                  | " "                         |
| Q76        | 0.1           | 10                  | " "                         |
| Q21        | 0.1           | 5                   | " "                         |
| Q22        | 0.1           | 5                   | " "                         |
| Q78        | 0.1           | 5                   | " "                         |
| Q79        | 0.1           | 5                   | " "                         |
| O98        | 0.1           | 1                   | Complete paralysis, 11 days |
| Q23        | 0.1           | 1                   | No paralysis                |
| Q85        | 0.1           | 1                   | " "                         |
| Q86        | 0.1           | 1                   | " "                         |
| Q25        | 0.1           | 0.2                 | Partial paralysis, 17 days  |
| O86        | 0.1           | 0.2                 | No paralysis                |
| Q66        | 0.1           | 0.2                 | " "                         |
| Q80        | 0.1           | 0.2                 | " "                         |
| Q57        | 0.1           | 0.1                 | Complete paralysis, 13 days |
| Q67        | 0.1           | 0.1                 | Partial paralysis, 10 days  |
| Q56        | 0.1           | 0.1                 | No paralysis                |
| Q81        | 0.1           | 0.1                 | " "                         |
| Q58        | 0.1           | 0.05                | Complete paralysis, 19 days |
| Q60        | 0.1           | 0.05                | " " 5 "                     |
| Q68        | 0.1           | 0.05                | " " 9 "                     |
| Q83        | 0.1           | 0.05                | " " 13 "                    |
| Q62*       | 0.1           | —                   | " " 7 "                     |
| O90*       | 0.1           | —                   | " " 7 "                     |
| Q26*       | 0.1           | —                   | Partial " 9 "               |
| Q54*       | 0.1           | —                   | Complete " 12 "             |
| Q84*       | 0.1           | —                   | " " 9 "                     |

\* Control monkeys receiving virus mixed with either saline or distilled water.

Monkeys surviving without paralysis were reinfected 1 month later. All developed typical paralysis, except Q82 which died on the 15th day without symptoms of poliomyelitis.

## DISCUSSION

The experimental data described in this report are interesting in several ways. First, they show that extraordinarily small amounts of vitamin C are capable of rendering non-infectious multiple paralytic doses of poliomyelitis virus. Second, they reveal a remarkable similarity in the quantitative aspects of this inactivation when compared with the neutralization of diphtheria toxin by vitamin C. The fact that two such heterogenous substances as diphtheria toxin and poliomyelitis virus, one a lifeless poison and the other a presumably living agent, should prove to be susceptible to the inactivating effect of no less than three highly reducing substances, *i.e.* adrenalin, cortin and vitamin C, all of which are present in the adrenal gland, seems to us particularly worthy of emphasis since it may serve to provide a common basis for a biochemical explanation of their destruction. The third, and undoubtedly most important, point of interest is to be found in the fact that vitamin C in the reduced form, as employed in these tests, is a normal constituent of various animal and human tissues, particularly adrenal and brain, and occurs also in a reversibly oxidized state in the blood (9). If the quantitative assays published in the literature are acceptable, the amounts of vitamin C normally present in the central nervous system are well within reach of the range found to be neutralizing in this paper. Further investigations will have to show whether the neutralization phenomenon observed when certain normal human and animal sera are brought into contact with poliomyelitis virus, is in any way influenced by the presence of vitamin C in the serum; also, whether the natural resistance of certain species of animals to intracerebral injection of this virus shows any correlation with the concentration of vitamin C in the brain or cord. Obviously, similar implications may be invoked for an explanation of the varying susceptibility of man to natural infection with poliomyelitis.

Experiments are now under way to determine whether vitamin C possesses any prophylactic or therapeutic properties in the treatment or prevention of experimental poliomyelitis.

## SUMMARY AND CONCLUSIONS

The experimental evidence presented in this paper shows that multiple paralytic doses of poliomyelitis virus, when mixed with very small amounts of crystalline vitamin C (ascorbic acid), are rendered non-infectious as determined by intracerebral injection of such mixtures into *rhesus* monkeys.

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# THE PROGRESSION TO CARCINOMA OF VIRUS-INDUCED RABBIT PAPILLOMAS (SHOPE)\*

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PLATES 19 TO 24

(Received for publication, June 28, 1935)

The cutaneous papillomas induced by a virus procured by Shope (1) from "cottontail" rabbits possess the traits whereby tumors are recognized (2). After they have grown for some weeks they frequently thrust processes into the tissue beneath them, sometimes entering the blood and lymph vessels. If even earlier they are transplanted to favorable situations within the host they may look and behave like epidermoid carcinomas, invading, destroying, and causing death. These observations, and the malignant aspect of sections from one of our rabbits and from one of Shope's, have led us to keep animals with actively proliferating papillomas consequent on virus inoculation, to learn whether they would become carcinomatous. This has happened in 7 domestic rabbits of 10 kept more than 200 days, while in an 8th animal a new tumor has developed of problematic malignancy. The cancers have been multiple in every animal, and metastasis has taken place in 5 of them,—to the regional lymph nodes in 4, to the retroperitoneal nodes in addition in 1, and to the lungs in a 5th instance. One cancer has been successfully transplanted.

## *Incidence of the Cancers*

The cancers have developed from the papillomas of domestic rabbits inoculated with extracts of the glycerinated growths of cottontails. Malignancy has never appeared in the latter, though 10 with induced papillomas have been under observation several months longer than the domestic rabbits. These were chosen for preservation because

\* Reported in abstract before the Society of Experimental Biology and Medicine, Jan. 16, 1935.

their papillomas enlarged rapidly and progressively. Others equally favorable, perhaps more so, succumbed early from complications (infection, hemorrhage, malnutrition) due to the large masses of proliferating tissue. As time passed the growths of some of the group enlarged more slowly or ceased to do so, and in one instance gradual retrogression took place. Eventually it became plain that the better the papilloma grew the more likely was cancer to occur and the more numerous were the situations of its occurrence. This held true not only of the growths in different rabbits but of those in a single individual. Breed and sex had no evident influence. All were young adults when inoculated.

### *The First Appearance of Malignancy*

The carcinomas appeared in the midst of papillomas more than 4 months old, which in most instances were still broadening and in all were building up tissue.

The new tumors were difficult to recognize early, when within the masses resulting from inoculation of broad areas. With the animal etherized, these masses could be turned for palpation; but often the irregular bosses on their under side, indicative of early carcinoma, could not be discriminated from the keratinized pearls frequent under old papillomas, though these were usually firmer and spherical. The malignant change was readily perceptible, however, in the papillomatous masses 1 to 3 cm. in diameter (Figs. 1, 4), consequent on tattooing virus into small spots. When vigorous, they became more fleshy as months passed, bulging laterally so that their bases appeared constricted. They had a squat, onion shape, or were surmounted by several dry, jagged peaks; and cross-section disclosed the usual vertical striation, with living tissue nearly a centimeter above the skin in many places, instead of only here and there as previously. Their bases, formerly smooth and thin, except for an occasional knob-like pearl, now felt thickened and convex, sometimes almost hemispherical. When sliced vertically the tissue bulged; the sooty pigmentation, often prominent before, had largely disappeared; and the demarcation from the underlying structures, once hair-sharp, was often blurred. The basal convexity was due to active proliferation at the lower ends of the vertical folds of papillomatous tissue, with a fan-like broadening in consequence. Often no further change took place for many weeks; but when the cancerous tendency was pronounced, the gray or cream-colored, vertically striated, dry peaks underwent gradual replacement by a low, rough expanse of flaky, irregularly striated, brownish material, obviously dried exudate in part. The skin at the edge of the growth became raised, ruddy, and tense, owing to its extension beneath the epidermis; and outside its long-established borders one or more spheri-

cal swellings often appeared, which rapidly matured into large, subepidermal pearls (Fig. 16) and later sometimes became projecting papillomas. These were localized extensions into new territory such as had never previously occurred.

Often before the growth reached this state, the animal had begun to gnaw it, and a firm mass could be felt extending downwards and sideways from its base. Biopsy here disclosed a carcinoma. In other cases the superficial tissue came, or was torn, away, leaving a raised, beefy disc (Fig. 18) or an ulcer with indurated walls (Figs. 1 and 6). Less frequently cancer developed under an intact papilloma (Fig. 7). Sometimes one growth out of many, or some special part of a large papillomatous mass, was gnawed for weeks before cancer became recognizable; but generally the gnawing was done afterwards. Ordinary papillomas are not gnawed as a rule unless their situation or size causes trouble. The development of malignancy was evidently attended often by annoying sensations, as in the case of human skin cancers (3).

Sometimes in the gross the cancer appeared to have arisen everywhere from the base of a papilloma resulting from punctate inoculation; but in most cases it was more or less central, appearing where the growth had existed longest, was deepest, and had most reactive tissue underneath it. The cancers appearing in the expanses due to broadcast inoculation, though multiple, implicated but little of the growth at first. The latter had usually become confluent only secondarily; and hence the relation of the cancers to its oldest parts could not be discerned. Embedded in the thin, underlying scar tissue were scattered pearls up to a centimeter across,—containing dry, creamy, lamellated material (keratinized scales) that readily shelled out,—and in addition, under and amidst the growth, discoid, nodular or hemispherical masses of close-textured tissue diversified with smaller pearls, yellow dots, or serpiginous necroses, and devoid of the palisade striation of the papilloma, and of the gray or sooty pigmentation so usual with it. These were the early cancers.

### *The Malignant Tumors*

At this writing more than 50 frankly malignant tumors have appeared in the 8 rabbits. Of these about 30 have been studied histologically,<sup>1</sup> while the nature of the others has been evident from their gross features. In addition numerous minute cancers have been procured by the biopsy of papillomas. Many of the new tumors have been malignant papillomas, the rest either squamous cell carcinomas or malignant papillomas in transition to these latter, or else and frequently, tumors representative of one stage or another of the change. Most of the new growths have enlarged steadily, though sometimes with quiescent periods; and they have caused the death of

<sup>1</sup> D. R. 2-53, killed recently, has provided 20 more.



3 hosts thus far, while 2 more have died of intercurrent ailments. Anemia has necessitated many transfusions; and local purulence has required drainage. In the gross two types of cancer could be discriminated,—fungoid, long localized and relatively benign, with a more or less definitely papillomatous character, and eroding, ulcerative, and metastasizing, squamous cell carcinomas.

The fungoid cancers took the form of projecting mounds or hassocks of ruddy, soft, vascular tissue, devoid of the fissures elsewhere present amongst the dry peaks (Fig. 18). When gnawed they were rapidly repaired. Slicing disclosed yellow streaks and dots and creamy pearls, amidst fine-textured tissue with few or no striae and these irregular and indistinct. The growth had penetrated the scar tissue mixed with fibrous corium, and extended laterally in the subcutaneous tissue as a firm, thick, pale layer variegated with the dots and pearls mentioned above. Sometimes it had a conglomerated aspect, like pudding stone (Fig. 22).

Instead of heaping up on the skin surface, the epithelium burrowed as irregular, tube-like processes (Fig. 19) amidst an abundant, reactive tissue in which subacute inflammation was frequent. The growth was notably desmoplastic, unlike the ordinary papilloma; but its epithelium resembled that of the latter more or less closely and tended to differentiate in the same way, with result in cores of keratinized scales. As the tubes grew down they branched or broke up into cell aggregates, some of which rounded out secondarily into pearls like those beneath an ordinary papilloma. Often instead the cells became necrotic, and a central debris accumulated, forming the dots and opaque strands noted in the gross. At the advancing edge of deep growths the invading processes were narrower, and not infrequently isolated cords and nests of cells were present as in a squamous cell carcinoma. The neoplasm penetrated amidst the cutaneous muscle and undermined, encroached upon, supplanted, and fused the neighboring papillomas. Extension tended to be slow, however, and metastasis did not take place.

The eroding type of carcinoma was sometimes recognizable early as a firm down-growth, rounded, nodular, or shaped like a flange or prong. More often though it first came to attention when the overlying, dry peaks fell off or were torn away, leaving an ulcer with necrotic or granulating surface, which extended later (Figs. 1, 4). Occasionally a narrow fissure lined with grumous material opened amidst the peaks, subsequently forming a deep crater. Ulcer and crater had walls of gristly tissue which soon encroached irregularly on the subcutaneous structures and skin, the latter becoming thickened, raised and nodular, fixed, glistening, and tense before it broke down. Adjacent papillomas were undermined, infiltrated, and destroyed. Section of them laid bare a shallow, raw, superficial layer, soft and pink, granulating or ragged, perhaps hemorrhagic or purulent, overlying a dense plaque of tissue that grated under the knife and was pale, close-textured, and more or less distinctly dotted with yellow. The growths enlarged in the loose, subcutaneous tissue without becoming fixed on the deep body wall. Secondary contraction often led to puckering of the skin, and drew neighboring papillomas to-

gether. Sometimes the cancer destroyed all those about it without occupying much more than half their space. Retraction of an overlying nipple was once observed.

Microscopically the eroding tumors consisted of nests, cords, and strands of invasive and destructive epithelial cells of squamous type (Figs. 2, 3). Anaplasia was sometimes great, and occasionally the condition approached *carcinoma simplex*. The growth induced a profuse formation of new connective tissue. It contained, like the fungoid tumors, some makrophages, plasma cells, a greater or less number of lymphocytes,—scattered or in small accumulations,—and some polymorphonuclear leukocytes, especially when there was bacterial infection. Foci of acute inflammation so caused were frequent, as also edema, small hemorrhages, and thrombosis within the numerous, wide, thin-walled vessels. The epithelium penetrated amidst and into the fibrous bundles of the deep corium, destroying or breaking them up; and it often entered and replaced the substance of muscle fibers. Extension into the lymphatics was common. A surface spread of the neoplastic epithelium soon covered gnawed spots, when these did not become purulent;<sup>2</sup> but infection with pus-producing organisms was the rule. Except for repeated transfusions nearly all of the animals would have died early. Metastasis to the axillary lymph nodes has been frequent (Fig. 4). In one case it attracted attention before the primary growth did (Fig. 7). In another the retro-peritoneal glands just above the pelvis were almost wholly replaced by metastases deriving from experimental implants of the growth in the thighs; and the neighboring adventitia of the aorta and vena cava were involved (Fig. 11). A lung metastasis was found in a rabbit free from local secondaries (Figs. 29, 30, 31).

All gradations between the fungoid and ulcerative, papillomatous and squamous cell, types of malignant growth were encountered (Text-fig. A), not infrequently within the same tumor (*carcinoma varia*), early biopsies disclosing tubular downward extensions which branched and broke up into the cords, strands, and cell groups typical of squamous cell carcinoma (Fig. 8).

### *Abstracts of the Case Histories*

W. R. and D. R. mean wild and domestic rabbit respectively; R. and L., right and left side; ax., axilla; P. or pap., papilloma; malig. pap., malignant papilloma; squam. carc., squamous cell carcinoma; metast., metastasis; inoc., inoculation; and V., virus (Tyrode extract of glycerinated pap.). The paps. of multiple inoc. are numbered. All biopsies were done under ether anesthesia. Materials for implantation were cut fine, suspended in Tyrode, and injected into the leg muscles.

Dutch "show" rabbits (D. R. 2-38 and 2-39) with white hair on front half of body and black hair on rear: tattooed on sides at numerous spots 2 mm. across, with V. from W. R. 18, May 3, 1934. The paps. developing in "black" regions

<sup>2</sup> The papillomas offered far more resistance to bacterial infection than the cancers, their epithelium keratinizing despite it.

were sooty, and those in "white" ones non-pigmented save for an occasional, thin streak. Further tattooings carried out 25 days later with V. 5-38 + 6-38 also gave growths. Tracings made at intervals.

**D. R. 2-38.**—17 pigmented and 4 non-pigmented paps. Bases of 2 pigmented, of first inoc., P. 2 and 5 R., infiltrated on 34th, 41st, and 46th days with Scharlach R in olive oil to stimulate proliferation (4). *132 days*: paps. at all sites. Those treated with Scharlach R not especially big, but all of first inoc. slightly larger than of second; each pap. an oval or circular group 1.1 to 2.6 cm. in diam., of jagged peaks cleft nearly to skin level. *210 days*: further progressive enlargement. *214 days*: dry peaks of P. 5, L. gnawed away, leaving ulcer with raised, nodular edges and plaque-like, firm base (Fig. 1). Sagittal section discloses squam. carc. (Fig. 2). Bits implanted in all upper legs (abscesses resulted). P. 5, R. has united with 4 and 6 R., induration exists beneath, and a fissure discloses grumous material. Similar fissure amidst coalesced P. 1 and 2, R. *237 days*: shotty lymph node removed from L. ax. Contains carcinoma like that of 5, L. (Fig. 3). Implantations in flexor thigh muscles. (These yielded progressively growing tumors.)

*249 days*: P. 1, L. gnawed; mass exists under and about it, with early skin involvement. Ulceration followed. The new tumor coalesced with P. 5 and the resulting large ulcer (Fig. 4), involved adjacent paps. *271 days*: the two cancers on R. form a common mass 7 x 4.5 cm. with deep central ulcers lined by yellow necrosis. Biopsy shows squam. carc. *277 days*: new nodule in L. ax. Cancers on sides now meeting under belly. *319 days*: growths and ax. nodule enlarging. *342 days*: all paps. on L. destroyed by the extending cancer except one which is ulcerating independently. *372 days*: firm nodule in R. ax. *382 days*: malignant masses are smaller, as also implantation nodules and ax. metast. Ulcerated pap. on R. removed. Sections show ordinary pap., malig. pap., and a distinct squam. carc. *397 days*: all growths dwindling. Biopsies now of implantation growth and ax. metast. show regressive changes (malignant cells cornifying *en masse* and dying).

*Comment.*—Animal still alive. 3 squam. carc. noted after 214 days, 2 of them in the only paps. injected with Scharlach R. 2 other carcs. appeared later. Metast. in regional lymph nodes, and implantation growths in hind legs. All now seem regressing.

**D. R. 2-39.**—9 pigmented and 5 non-pigmented paps. *40th and 46th days*: base of P. 4, R. in black region infiltrated with Scharlach R. *132 days*: all paps. except 4, R. are raised, fissured discs, some closely adjoining. P. 4, gnawed nearly flat, and indurated plaque extends from beneath it into skin.

*211 days*: P. 4, R. now a granulating ulcer with gristly, rolled border (Fig. 6). Biopsy shows malig. pap. changing to squam. carc. (Figs. 6 A and 8). *230 days*: growth has extended under and involved neighboring paps. Biopsy (Fig. 6, B) of its furthest subcutaneous extension; same gradations from malig. pap. to squam. carc. (Fig. 9). Bits implanted in all upper legs posteriorly. *242 days*: rapidly enlarging nodules in legs; biopsy shows invasive squam. carc. (Fig. 10).

Bits implanted in ant. thigh muscles of host (squam. carc. here later), and into 3 other Dutch D. R. (negative results). Leg tumors from first implants now large. Shotty nodule in R. ax. Carc. on side has destroyed all 7 coalesced pigmented paps. save one (P. 7). This is indurated, gnawed; biopsy discloses independent squam. carc. The anterior masses resulting from coalescence of non-pigmented P.'s on R. and L. have been gnawed nearly to the thickened bases.

253 days: all P.'s on L. have thickened, bulging bases. 264 days: cancerous mass on R. steadily enlarging. 288 days: greatly emaciated; fleshy nodules in skin next large ulcerated mass on R., with puckering from secondary contraction. 293 days: scrotum, hind legs, and belly edematous; very weak. 298 days: moribund; sacrificed. Bits of cancerous mass on R. and of implantation tumors deriving from it, implanted in legs of 9 Dutch rabbits. In one a nodule appeared, 1.2 cm. across after 79 days; squam. carc. on biopsy (Fig. 27).

*Post-Mortem Findings.*—Large, ulcerated squam. carc. in skin and subcutaneous tissue on R., replacing pigmented P.'s. Non-pigmented P. mass is in pre-cancerous state (Fig. 16), as is mass on L., and this contains a minute squam. carc. (No pigmented P.'s on this side.) Nodular metasts. in several R. ax. glands, and in retroperitoneal glands. Here the squam. carc. is wholly anaplastic (Fig. 11), invading walls of aorta and vena cava. At all implantation sites irregularly globoid squam. carc. up to 7.5 cm. in diameter, some with purulent infection.

*Comment.*—Malignancy already advanced in one pap., when noted on 132nd day after V. inoc., and 116th day after growths appeared. Base of the cancerous pap. had been twice infiltrated with Scharlach R long before. Becoming a malign. pap. then a squam. carc., it underwent further anaplasia in the implantation nodules and metastases. At death another large and several small squam. carc. were present.

June 11, 1934: two Dutch "show" rabbits, D. R. 2-52, -53, and an albino, D. R. 2-50, tattooed on sides with V. 5-39 + 6-48. Paps. appeared within 3 weeks, enlarging progressively: creamy or sooty in white or black regions respectively of Dutch animals, all creamy in albino.

D. R. 2-52.—175 days: The 15 sooty and 10 pale paps. are jagged masses 1.5 to 2.5 cm. across, some fused. P.'s 5, 6, and 7 R. recently gnawed, exposing fungoid growths about 1 cm. high, with vertical sides (Fig. 18) and bulging, deep bases. Slice of P. 7 on 176th day shows a tubular neoplasm (malign. pap.), deriving from ordinary pap. (Fig. 19). 185 days: biopsy of subcutaneous extension under normal skin: same transitions to malign. pap. Bits implanted in hind muscles of all upper legs. 189 days: all paps. suddenly more fleshy. 198 days: fungoid growths enlarging: shotty lumps in legs.

210 days: Slices of P.'s 6 and 14, L. show invasive Shope pap. only (Fig. 17). Bits from P. 14 implanted in flexors of thighs. 219 days: P.'s 5 and 7, L. gnawed. 231 days: P. 5 L. enlarging, thick based. 242 days: all paps. enlarging rapidly and coalescing; fungoid growths 6 and 7 R. have common, gristly base. Nodules at implantation sites enlarging. 267 days: thick, gristly layer has extended from

base of 5 + 6 + 7, R. under nearly all paps. on R., involving them. 273 days: subcutaneous implantation growth involving skin excised from R. thigh (mixture of ordinary and malig. pap.). 280 days: P. 5 and 7, L., long separately fungating, now united, with deep base.

During subsequent period to death on 344th day growths of each side completely coalesced, forming masses with fungoid areas. Cancers appeared where bases of P. 6 and 14 respectively had been cut through, one a malig. pap., the other squam. carc. on biopsy. An ulcer with firm, deep base replaced P. 6 L., and a flange and a prong, both gristly, extended down from P. 3 R. and P. 1 L. respectively. Section of the prong showed malig. pap. The irregularly globular, implantation masses became 5 to 7 cm. in diameter, and one had to be drained of pus. The emaciated animal was killed when moribund.

*Post-Mortem Findings.*—On each side, numerous paps. and cancers, some ulcerated, fused into a mass (Fig. 22). Of 9 tumors examined histologically, 2 were small squam. carcs., 2 cystic growths (Fig. 5), and 5 large malig. paps. Bits of one of these last, implanted in legs, yielded similar growths.

*Comment.*—Most of the new tumors were malig. paps., of which many were recognizable besides those biopsied. The implantation growths retained this character. No metast.

**D. R. 2-53.**—The 5 discrete, pale paps. and 16 sooty enlarged slowly until about the 260th day, then becoming stationary, 1 to 2.5 cm. across, not fleshy, bases superficial. 285 days: blunt forceps accidentally thrust into base of 5 R., with hemorrhage. Paps. have begun to grow again in some instances, notably 5 R. One removed on 295th day was 2 cm. across, fleshy, bulging downwards but still only a Shope pap. Shotty nodule now in L. ax. 306 days: P. 5 R., recently injured, is now fungoid, encroaching on P. 11. 316 days: nodule enlarging in L. ax. and primary tumor now perceptible as cone-shaped growth downward from the fleshy, newly coalesced P.'s 3 and 4. 329 days: prong under P. 3 + 4 removed (malig. pap. becoming squam. carc.), and also L. ax. nodule (Fig. 7)—(squam. carc. with some features of malig. pap.). Bits of former implanted in upper forelegs. 336 days: fungoid growth has replaced P. 5 R. (Biopsy shows squam. carc. arising directly from Shope pap.)

*Comment.*—Still living. For months paps. grew slowly, then some of them rapidly, and squam. carc. appeared late in one of these on each side, soon metastasizing to regional nodes. One pap. in which cancer appeared had been injured mechanically.

**D. R. 2-50, Albino.**—The 18 paps. grew slowly, were 2 cm. across by the 220th day, very superficial, all gnawed. They remained stationary for a few later weeks and then dwindled, about half disappearing and the rest only 0.2 to 0.7 cm. across when the rabbit was killed on the 281st day. Sections showed retrogression of the usual orderly sort (4).

On May 16, 1934, V. 5-38 + 6-38 was rubbed into a scarified skin area about 5 by 7 cm. on one side of 3 brown-gray rabbits (D. R. 2-47, -48, and -49), and Cliff-

Peck V. into a like area on the other. Confluent growths appeared very early, within 8 to 12 days.

D. R. 2-49.—The broad pap. masses became several cm. high in early months; later gnawed low, fleshy, and inflamed. They were about 7 by 7 cm., and 1 cm. high, covered with dry, serosanguineous exudate, at death from diarrhea on 212th day. Near middle of mass caused by V. 5-38 + 6-48, elsewhere vertically striated and sharply defined, was a discoid patch 3 cm. across, fungating, veal-like, with ill-demarcated base. Section showed malig. pap. grading downwards into squam. carc. 2 smaller, similar tumors, widely separate, and a 3rd large, deep and plaque-like, in mass due to Cliff-Peck V. All were squam. carc., in some spots replacing muscle. No metast.

D. R. 2-48.—By 116th day had vigorous growths about 6 by 8 cm.,  $1\frac{1}{2}$  to 3 cm. high, raw and beefy from gnawing. When covered with a binder they rapidly built up to 5 cm., dried, and became so heavy that repeated paring was necessary. 182 days: growth on L. caused by V. 5-38 + 6-38 incised under ether anesthesia to drain pus pockets; large, underlying abscess also evacuated. 240 days: scarring has reduced L. growth to 3.5 cm. diameter, but it is still building up actively. 251 days: a smooth, ruddy mound, like a *glans penis*, has appeared amidst mass on L. Biopsy shows florid pap. of questionable malignancy with plexiform base. This by 275th day had nearly replaced mass; it was still encephaloid, superficial. 384 days: tumor on L. unchanged in gross (Fig. 28) and microscopically, on new biopsy (Fig. 21). Ordinary pap. on R. somewhat smaller than before. Recent gnawings of latter, with infection and purulence, have not been followed by cancer.

*Comment.*—Localized purulence and incision of a vigorous pap. mass was soon followed by a new tumor of questionable malignancy. The other pap. mass stopped enlarging and has not turned cancerous during several months, though latterly purulent.

D. R. 2-47.—Paps. appeared more slowly than in others but formed confluent masses secondarily, becoming stationary after about 150 days and soon beginning to retrogress. Masses were still about 8 by 6 cm. and nearly 2 cm. high when rabbit died on 216th day. Sections showed orderly, thin-based pap., still proliferating in some regions.

D. R. 2-35.—April 30, 1934, Chinchilla inoc. on broad area of both sides with V. 5-38 + 6-38. 16 days: beginning, confluent papillomatosis. A Tyrode extract (0.1 per cent) of rabbit testicle frozen on 4th day of infection with vaccine V., New York Board of Health strain, was injected in L. expanse, 0.1 cc. at two widely separate points, and also into normal skin nearby: excellent "takes" with local necrosis and healing. The regions of vaccinal change were charted, and on 23rd and 24th days 4 slices removed from them. The pap. masses gradually became jagged mats, about 9 by 6.5 cm. and 2 cm. high on 212th day.

228 days: rounded, smooth bosses up to 1.5 cm. diameter felt under the masses, —pearls. 5 days later L. growth gnawed over area 3 cm. across, disclosing

raised, beefy tissue, devoid of clefts. Biopsies showed squam. carc. here, and elsewhere ordinary papilloma and a pearl, respectively, with beginning invasive extension from this last (Figs. 25, 26). Prompt healing, with replacement by same tissue as before. Animal kept beefy area raw; and after several weeks it gnawed region of previous vaccinia necrosis on R. for first time. Here on 244th day it exposed a fleshy disc. 1.8 cm. across, demarcated by a fissure (Fig. 29). Biopsy showed squam. carc. 286 days: died during operation.

*Post-Mortem Findings.*—In R. mass 2 large squam. carcs. and a cystic tumor (Fig. 14). In L. mass 2 squam. carcs. and a malig. pap. (Fig. 20). Numerous early malignancies (Fig. 15) elsewhere in pap. masses, and many pearls beneath. One lung metastasis with morphology of largest squam. carc. on R. (Figs. 30 and 31).

*Comment.*—One of the large, early cancers appeared at the site of a healed vaccinia lesion in the pap. expanse.

D. R. 2-05.—Brown-gray, inoc. with V. 5-38 + 6-48 in broad area on each side May 4, 1934. Confluent, jagged masses about 12 by 8 cm. developed, later changing to solid, cutaneous horns about 6 cm. across and 5 cm. high, building up actively. On the 190th day L. horn was gnawed, and palpation disclosed a small, gristly mass under base. This enlarged, horn was gnawed away entirely, and at death on 348th day a great ulcerated mass like a truncated cone was present, 10 cm. across, 3 cm. deep, with its base in the subcutaneous tissue and a few small pap. peaks along its border. The bulk of the mass proved to be malig. pap. tissue, with cysts, like those of D. R. 2-52 (Fig. 22), and transitions to anaplastic, squamous cell carcinoma. The superficial paps. were of the "second order" (4). The axillary nodules were metast. of squam. carc., containing cysts (Fig. 24) reminiscent of the papilloma.

The horn on the R., long unchanged in size, had continued to proliferate, and latterly 2 subepidermal pearls had extended from its base. It was a pap. of the second order, with beginning squamous cell carcinomatosis and malignant papillomatosis at separate locations in it.

*Comment.*—The cancer was solitary for many weeks, but at time of death others were appearing in the mass on the opposite side.

Two instances from our previous papers (4, 5, 7) should be added, of what now appears to have been indubitable carcinosis (D. R. 1-2; Dr. Shope's rabbit).

### *The Progression to Malignancy*

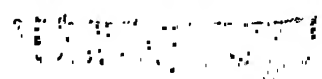
The early stages of the cancerous change cannot be comprehensively described without inclusion of the entire course of events in vigorous papillomas. These tend toward malignancy from the beginning, and attain it by a continuous series of alterations (Text-fig. A).

ny vigorous Shope papilloma

Cystic papilloma  
(D.R. 2-35a; D.R. 2-52 f)

1 Epidermal level

4



Malignant papilloma  
(D.R. 2-52 a,b,c,d,e)

Squamous cell carcinoma  
deriving from malignant papilloma  
(D.R. 2-39)

5

6



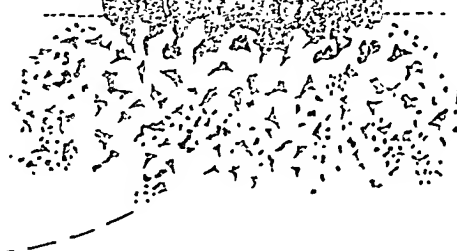
More immediate derivation

(D.R. 2-35 b)

with progressive anaplasia  
(D.R. 2-38 a)

7

8



TEXT-FIG. A. The progression to carcinoma: schematic drawing. The numbers in brackets refer to rabbits in which tumors illustrative of one state or another of the progression were encountered, and the added letters designate the individual tumors of each animal. Many growths underwent further morphological changes after they had become malignant, a fact indicated by the arrows.



The developing papillomatosis has already been described by Hurst and by ourselves (6, 7). The epithelium grows down but soon meets the fibrous corium and a folding outwards ensues. For weeks the growth is very superficial, with an almost linear basal demarcation: the hair follicles and sebaceous glands are merely overlaid, not destroyed. Gradually it becomes bedded somewhat more deeply, and the skin appendages disappear. As early as the 2nd month blunt processes, composed of ill-ordered epithelial cells, may be pushing down between the collagen bundles (Fig. 12; Text-fig. A, 2). Their advance is slow, and the cells tend to differentiate and keratinize like those of the surface growth, with result in pearls, either separate from the surface mass or connecting by a bottle neck. They consist of concentrically arranged, exfoliated scales deriving from a layer of living epithelium that has papillomatous features (Fig. 13). Nearly all are cream-colored, even when the surface papilloma is sooty black, a difference not referable to their situation,—since implantation nodules are often pigmented,—but to the activity of the burrowing cells. (The eventual cancers were always wholly unpigmented though deriving usually from gray or black papillomas.)

As time passes scar tissue of greater or less density and thickness, depending upon intercurrent influences (trauma, bacterial infection), forms beneath the papilloma; and the fibrous corium is incorporated therein. Localized edema and focal inflammation are now frequent; and, in proportion as they occur the papilloma becomes disorderly, with thick and thin basal foldings, unevenly ranked (Fig. 13, Text-fig. A, 3). More pearls form, sometimes a centimeter down, and they may be irregular in shape, with broad epithelial processes pushing out from them. In such processes, as at some situations in the parent growth, the cells may have largely lost polarity, and they and their nuclei may be distinctly larger than before, their shape more variable, and pathological mitoses not infrequent. The picture now suggests beginning carcinomatosis. The surface mass often seems to have ceased its extension; yet it still builds up actively, material lost from its dry summits being soon replaced; and successive tracings show that it is slowly enlarging. Growths which interfere with posture and movement are gnawed and become infected and fungoid,—great beefy masses, several centimeters high, which bleed easily. If protected with a binder these dry and assume the usual aspect.

The subsequent events vary considerably. We have made numerous biopsies to study them. Sometimes the disorder of the papilloma everywhere increases, its basal foldings become crowded and still more irregular, and certain of them, extending down, anastomose and branch secondarily with result in a plexiform network two or three millimeters deep. The basal limits of the growth cannot then be made out in the gross. Occasionally the invasive processes branch almost regularly with result in a "papilloma of the second order" (4). Frank malignancy is attested by a further extension with tissue destruction. In one animal (D. R. 2-48) the new tumor, though replacing the ordinary papillomatous mass, and very different from it in the gross (Fig. 28), has remained superficial for more than 5 months and of dubious malignancy. Histologically (Fig. 21) it exemplifies the precancerous disorder.

In other cases an especially vigorous proliferation takes place at certain spots in

the papillomatous layer, with result in one or more discrete, non-pigmented "onions" in the midst of a jagged, sooty expanse. Small epithelial pearls develop in and under the "onions," and downgrowth takes place secondarily from the wall of one or several of these (Fig. 15), perhaps with later dispersion of the epithelium into the nests and groups characteristic of squamous cell carcinoma. Closely akin are cases in which subepidermal pearls form outside the long established border of a papilloma (Fig. 16), sometimes turning carcinomatous secondarily, the epithelial cells becoming large and disorderly, with big nuclei, pathological mitoses, and frequent giant or multinucleate elements. The alteration may be local or occur everywhere around the wall of the pearl, and it is followed by disorderly aggression.

In still other instances the cellular changes mentioned take place in the basal foldings of a papilloma that has not as yet encroached on the neighboring tissues, though growing vigorously, and the result is an easily recognizable cancerous focus, still within the confines of the papilloma, and grading into the latter morphologically.

When cancer has occurred it may long remain quiescent and minute, or it may rapidly replace the papilloma. Biopsy at this time of a discrete, bulging, onion-shaped growth may disclose dry, overlying papilloma and a living, basal layer composed of carcinoma, giving the false impression that the malignant change has been a general one. An earlier section would have shown its origin to be local, though often at several spots.

Fortunately for the study of the cancerous changes these find expression not only in a graded progression from ordinary papillomatosis, but in tumors representative of one stage or another of the entire process (Text-fig. A). One such instance has already been described (Figs. 21 and 28). Fig. 14 shows a vertical section through an irregular nodule found beneath a papillomatous mass: the papilloma has penetrated the scar tissue at one point and ramified, forming numerous cysts. These cysts are like the pearls found beneath papillomatous masses at an early period; but in the present instance serial sections have shown that narrow tongues of aggressive epithelium extend from them deep amidst the voluntary muscle fibres. In Figs. 5 and 22 a similar, cystic growth has extended far out under the normal skin. The way in which such growths begin is evident from one in which the downward penetration had just started at the time when a biopsy was done. A papilloma 210 days old (D. R. 2-52) had become notably fleshy, bulging downwards. Biopsy showed that at the center of the base, where pressure was presumably greatest, the growth, though still orderly, had advanced into the dense scar tissue (Fig. 17). It is questionable whether these cystic growths (Text-fig. A, 4) can be deemed malignant.<sup>3</sup>

Decision is easy with the tumors representing the next stage in the morphologi-

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<sup>3</sup> In D. R. 2-53, killed since this paper was concluded, a metastasis from such a growth, with the same cystic morphology, has been found in an axillary lymph node.

cal progression. Fig. 20 shows in cross-section a persistently gnawed spot in a broad, papillomatous mass. Here a papillomatous downgrowth exists, ramifying and forming cysts filled with the debris of untimely cell necrosis. The growth has undermined and raised the neighboring, pigmented papilloma. Its initial relation to the latter can no longer be discerned.

This is malignant papillomatosis (Text-fig. A, 5). Another example has been figured with the fungoid carcinomas (Figs. 18 and 19). Here epithelial tubes are burrowing amidst new-formed connective tissue. Some of the epithelium is of the ordinary papillomatous sort, differentiating often into pearls; but much is notably anaplastic, and many of the pearls it forms contain amorphous debris. The growth eventually extended as a thick layer under some of the other papillomas on the rabbit's side, coalescing with similar growths that had arisen in these, and eventually forming one mass (Fig. 22). Bits cut from its base at an early period and implanted in the legs gave rise to large tumors, in which all gradations from benign to malignant papillomatosis were present, precisely as in the parent growth.

Here the state of malignant papillomatosis was not exceeded, despite the shuffling of opportunities effected by operation and transplantation. More often further alterations took place to squamous cell carcinoma (Text-fig. A, 6). Fig. 8 is from the fleshy border of the earliest cancer to appear in D. R. 2-39 (Fig. 6): here to all appearance a malignant papilloma is changing to squamous cell carcinoma as it grows down. But another explanation suggests itself, namely that the apparent transformation is due merely to stimulation by the cancer of the overlying papillomatous tissue. This alternative was ruled out by biopsy of the tip of an extension of the tumor far out beneath normal skin. Again the transition forms were present (Fig. 9).

Not only may benign papilloma change to malignant papilloma and this in turn to squamous cell carcinoma, but a further progression may take place to a state of ultimate malignancy, so to speak, in which every cell acts for itself (Text-fig. A, 8):—

Some of the material seen in Fig. 9 (second biopsy, D. R. 2-39), containing transitions from papilloma to carcinoma, was cut fine and injected into the leg muscles of the host. One of the resulting nodules was removed on the 12th day after implantation. It consisted of squamous cell carcinoma (Fig. 10), though with some indications (in abortive cyst formation) of its derivation from papilloma. The growth had evidently gained in malignancy. When the rabbit died metastases were found in the axillary and retroperitoneal lymph nodes, in the latter presumably as disseminations from the large implantation tumors in the hind legs, since the inguinal glands draining the skin cancers were uninvolved. The malignant epithelial cells showed little differentiation, had almost wholly replaced the retroperitoneal glands, and were individually invading the adventitia of the nearby vena cava, and aorta (Fig. 11).

In this instance the entire gamut of changes from ordinary Shope papilloma to malignant papilloma, and thence in turn to the most dishevelled and aggressive

of squamous cell carcinomas, was traversed at long last. Sometimes, on the other hand, the change to a squamous cell growth is soon over, and occupies but a few millimeters of tissue (Text-fig. A, 8). But even in such instances it comes about by graded alterations (Fig. 23).

### *The Influences Precipitating Carcinosis*

Some virus strains induce papillomas which sooner or later retrogress, whereas others yield growths that almost always go steadily on. Since cancer develops only when this happens, one must conclude that the virus strain can be of decisive importance, though no differences in carcinogenic effect have been perceptible in our experience with those strains which induced vigorous papillomas.

Among host influences the animal species is paramount, and individuality is frequently decisive. The general and local characters of the skin are of notable importance.

The virus affects rabbits only. Cottontails are its natural hosts. Their papillomas grow more slowly than those of domestic animals, are remarkably superficial, and after some months cease to enlarge though continuing to proliferate. A shallow layer of scar tissue now underlies them and this sometimes contracts, bunching the dry peaks. Retrogression is frequent. A group of cottontails with papillomas induced by virus strains carcinogenic for domestic rabbits have now been under our observation for more than a year. In none has cancer developed; nor has it come to Shope's attention in his numerous rabbits with naturally occurring papillomas.<sup>4</sup>

The papillomas induced at one inoculation in domestic rabbits of a single breed may differ much in their course, some going on to cancer and others becoming stationary or retrogressing. Save in exceptional instances (D. R. 2-53) the multiple papillomas of any one rabbit all behave in the same way; and one must conclude that they are influenced by some general condition, and that this can alter (D. R. 2-53).

Those rabbits which have skins most responsive to Scharlach R are the ones in which the papilloma grows best (4), providing the greatest chance of carcinosis. The malignant change is prone to occur in pigmented papillomas. In 4 Dutch rabbits 57 discrete, sooty papillomas were induced in skin carrying black hair and 24 pale ones where it was white. Of the former growths 13 became cancerous,—but 3 had been repeatedly stimulated by the injection of Scharlach R and hence must be excluded from consideration. Cancer developed in only 2 of the papillomas of white regions; and no certainty exists that in these it did not arise from melanotic tissue, of which occasional fine streaks were present.

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<sup>4</sup> Personal communication.

When the general disposition of the host (its species), its individual disposition, and its soil (the cells acted upon by the virus) were all favorable to papillomatosis and hence to cancer, this still occurred at some spots and not at others. One reason was occasionally evident in the differing behavior of the papillomas of the same animal (as *e.g.* D. R. 2-53, Fig. 7). Those which grew best, becoming fleshy, underwent malignant change. Here again, presumably, the factor of soil came into play, the virus at some spots affecting cells that were especially favorable to it, and hence to malignancy.

Vigorous papillomas eventually arrive at a condition in which carcinomatous changes may ensue at numerous situations within them. Yet the changes are always local at first, though the localities be many. Often the history suggests that intercurrent factors have had much to do with where and when malignancy developed.

Scharlach R was injected into 3 pigmented papillomas of the 26 induced in D. R. 2-38 and 2-39. The dye has a marked stimulating effect on such growths (4) but this is transitory; and at the time when frank malignancy developed, the injected masses were no larger than some of the others. Cancer appeared in all 3 however, whereas in only 3 of the other 23 growths; and one of the cancers arising in an injected papilloma was by far the earliest to appear.

The base of P. 5, R. of D. R. 2-53 was accidentally pierced on the 285th day. Cancer appeared 21 days later; but it was noted in another of the 15 pigmented growths at about the same time.

Vertical slices were taken on the 210th day through 2 of the pigmented papillomas of D. R. 2-52. They disclosed aggressive papilloma only (Fig. 17). The gaps soon filled up with tissue of this sort and discrete cancers developed there later.

Vaccine virus was injected early at two points in a broad papillomatous area of D. R. 2-35, causing necroses about  $1\frac{1}{2}$  cm. in diameter, which were duly charted. Replacement with papillomatous tissue was prompt. Months afterwards the animal tore away the papillomatous peaks at one of the charted spots, disclosing a squamous cell carcinoma.

In and under a large area of active papillomatosis on D. R. 2-48, pus pockets developed, necessitating drainage on the 182nd day. About 2 months later, a fungating tumor appeared in the mass and within a few weeks had replaced it wholly. A similar papillomatous area on the other side of the animal did not become purulent then or malignant later.

From a papillomatous area of D. R. 2-35, in which one cancer had appeared, as did several later, a large pearl was removed by exploratory operation. Everywhere save at one spot its epithelial lining appeared benign (Fig. 25); but here it was thickened and disordered, and had invaded the neighboring tissue (Fig. 26) which here and here only was inflamed and edematous, with scattered pus cells and diplococci. Elsewhere the epithelium had keratinized in concentric layers,

but here it had for some time been dying early, as the markings of the necrotic material attest.

Some of the growths of D. R. 1-22 were subjected to experiments that rendered them invasive (Scharlach R injections, layering with collodion, transplantation to the muscles, subcutaneous tissue, and viscera) (4). At death, after less than 3 months in all, many of them appeared malignant, and a large nodule with the morphology of a squamous cell carcinoma was present in a regional gland.

These examples indicate that local interferences can precipitate cancer in papillomas disposed to the change; yet such influences are far from doing so always, and cannot be deemed essential. Though locally favoring conditions bring on cancer in susceptible hosts at some spots prior to others, its rapidly increasing multiplicity as time passes shows that it would eventually occur anyhow. The effect of local factors approaches the crucial only in papillomas of slow growth, which might not become cancerous in the absence of promoting influences (*e.g.* D. R. 2-48). When the cancerous change takes place under such circumstances the growth rate may quicken so abruptly as to suggest that the malignant activity has been touched off in some way, as indeed it has.

All of the various interferences bringing on manifest cancer give rise to disturbances of the connective tissue underlying the papilloma. This association is no fortuitous one. Papillomas which remain well-ordered and to all appearances benign for long periods have always a thin connective tissue base. Those on the other hand which burrow (Fig. 13) and give other signs of beginning malignancy have inflamed, proliferating, and edematous bases. That the condition of the supporting connective tissue greatly influences the behavior of the papilloma is further indicated by experiments (7) in which implants infected with bacteria causing reactive connective tissue disturbance grew like epidermoid carcinomas, whereas in the absence of such disturbance the papillomatous aspect was retained.

It is a truism with clinicians that bacterial infection of a tumor is often followed by malignancy or enhances it; and the influence of connective tissue disturbances to further invasion has been generally recognized. The rôle of these factors must not be overstressed in the present relation, however. The diversity of the influences which precipitated malignant activity in the papilloma clearly indicates

their non-specific character. Sometimes cancerous downgrowth took place into dense scar tissue at spots where no locally favoring condition could be discerned. The trend of the papilloma toward malignancy evidently brings it at length to a state in which cancer is inevitable. Whether local influences are primarily responsible for this trend is another matter.

#### DISCUSSION

The progression to malignancy of papillomas is a common pathological event. The skin papillomas induced with tar and other agents in man, the rabbit, and the mouse frequently become carcinomatous, as do also papillomas of the human mouth and tongue. Laryngeal papillomas are supposed to undergo cancerous changes often, but proven instances are rare (8). The alterations leading to carcinoma in the tumors mentioned are the same, generally speaking, as in the Shope papilloma, and trauma, infection, chronic inflammation, operation, and other intercurrent influences frequently precipitate malignant activities, as in the case of this growth.

Papillomas of the human bladder, though deriving from epithelium of transitional type, provide many parallels with the Shope tumor. They trend toward carcinoma with such constancy that some authorities, notably Zuckerkandl (9), hold them all to be potentially malignant. Incision and local inflammation often bring on carcinosis. While still histologically benign the papillomas, like the rabbit growth, are readily transplantable in the host, as operative dissemination to the wall of the bladder only too often attests. Not a few cases are on record in which transperitoneal removal of an apparently benign papilloma has been followed by the appearance of implantation nodules in the healed laparotomy wound, without bladder recurrence (10). Such secondary tumors may appear benign, though they are more often malignant.

The rabbit cancers always arose from the papillomatous epithelium, not from such skin appendages and epidermal cells unaffected by the virus as underwent inclusion in the proliferating mass. This was plain not only from the way they originated (Text-fig. A) but from their morphological characters. There were no signs of a diversity of origin such as tar tumors of the skin exhibit, no growths referable to the hair follicles or sebaceous glands, and indeed no basal cell epitheliomas. The malignancy was consequent on changes in but one

kind of cell, namely that stimulated by the virus,—which is effective upon epidermal cells only (7); and the new tumors, though apparently various, were the manifestations of progress in a single direction, namely from papillomatosis to squamous cell carcinosis. From the experimenter's point of view the Shope virus is more than a notably effective carcinogenic agent.<sup>5</sup> By affecting cells of a single sort in the way that leads to malignancy it gives rise to what may be termed pure strain cancers. These should provide a controlled material for studies of the influence of accessory factors on the origin and manifestations of carcinosis.

The cancers developing from the rabbit papillomas were all acanthomas, tumors such as follow upon a great variety of skin irritations, most of them automatically excluded by their nature from functioning as the immediate cause for the malignancy. Tar, weak hydrochloric acid, Roentgen rays, and the bacilli of lupus can none of them be considered directly responsible for cancer, though all produce lesions in which it may develop. The Shope virus might be dismissed as acting merely in this way, did it not give rise to growths which themselves have the traits of tumors, which possess some malignant potentialities at an early period, and become carcinomatous by continuous alterations of form and behavior.

Shope could not at first recover virus from the papillomas engendered in domestic rabbits; but latterly he has done so, transmitting the disease by its means in 10 successive groups of animals (12). The rabbits of the present work were inoculated with strains known to be irrecoverable, but it has been possible by indirect means to demonstrate the presence of virus in the papillomas. A principle neutralizing it appears in the blood of rabbits carrying the growths (1). With Dr. J. G. Kidd as collaborator, we have titrated this principle,—wholly lacking in the normal animal,—and have found that its time of appearance and rate of increase vary directly as do those of the papillomatous mass. The amount of antigen, of virus that is to say, evidently becomes greater as the proliferating mass enlarges. The con-

<sup>5</sup> In D. R. 2-53, killed recently, after 370 days, a sagittal section was taken of each of the 20 discrete, papillomatous growths. All proved to be still Shope papillomas in greater or less part, 5 of them wholly such. Deriving from the other 15 were 20 distinct carcinomas.



clusion seems justified that the virus accompanies and is responsible for the characteristic epithelial proliferation so long as the growth remains a Shope papilloma. Attenuation experiments have shown that its state finds a direct reflection in terms of papilloma behavior; and the better the growth, the more likely is cancer to occur. The problem of causation narrows to the period when cancer begins. But when does cancer begin? Upon this point the morphology and behavior of the changing tumors yield no decisive information. In all save the most anaplastic of the cancers the influence of the Shope virus finds some expression still, in papillomatous features and cyst formation (Text-fig. A).<sup>6</sup> Often the alterations which lead to carcinosis do not stop when malignancy has been achieved, but go further until a state of great anaplasia has been attained. The postcancerous changes appear to be no separate course of events but only a continuation of what was long since begun.

These facts might be taken to indicate that the virus is the immediate cause for the carcinosis; yet they are compatible with the assumption that it merely provides an essential, preliminary, cell disturbance. The failure of cancer to appear when the papilloma is no longer progressing can be likened to the failure of tar cancer to develop when tarring is not kept up. The proximal cause for the carcinosis may conceivably be present, or effective, only in papillomas that are doing well. The special liability to cancer of those rabbits which are most favorable to the papilloma can be matched by the individual differences exhibited by tarred rabbits or mice as concerns papillomatosis, and the ensuing carcinosis. In them as in our rabbits cancer develops with special frequency from pigmented skin. The difficulties of telling precisely when cancer has supervened upon Shope papillomatosis are great, because it derives from a growth of neoplastic morphology; but they are considerable with irritation acanthomas in general. All authorities upon the "precanceroses" stress them.

The potential malignancy of the papillomas at an early period, as demonstrated experimentally, has not necessarily a large significance, for normal epithelium can be stimulated temporarily to malignant

<sup>6</sup> This is not surprising, since extraneous viruses flourish after their introduction into transplantable tumors (11), and some of them induce characteristic morphological changes (inclusion bodies).

behavior with Scharlach R or Sudan III. In the case of our young papillomas acting malignantly (7) there had been accessory stimulation. None of the growths stimulated to invasion and destruction at an early period, has given rise to secondaries, save perhaps D. R. 1-22 (*q.v.*).

The Shope papilloma frequently retrogresses after doing well for a time, whereas the cancers deriving from it and recognizable in the gross progress in most instances, though whether in all is questionable (D. R. 2-38 *q.v.*).<sup>7</sup> During the months before cancer appears a natural selection of individuals favorable to the papillomatosis, and in consequence to malignancy, is taking place. The longer the papillomatous growth endures the less is the incidence of retrogression; and one would expect it to be smaller still in the late period when cancer is present. In all of our animals developing cancers the uninvolved papillomas have continued to proliferate.

Whatever the immediate cause for the cancers, they are beyond question due primarily to a virus, and they develop from the papillomatous growth to which this gives rise. Are there other instances of the sort? We have been able to find none in the literature on the domestic animals, though virus-induced papillomas are frequent in dogs and cattle. Human pathology, however, provides a parallel that is remarkable in many ways.

*Condyloma acuminatum* is due to a filter-passing virus (13), but the occurrence of the growth under natural conditions depends both on individual susceptibility and on local irritations such as are produced by pathological secretions. When these last are done away with the growths ordinarily disappear. They are papillomas, branching as the rabbit papilloma does not, with thick-layered epithelium of squamous type, sharply demarcated from the connective tissue, and manifesting none of the early, invasive activities of the Shope tumor. The growths are not only benign but their presence is dependent on accessory conditions. Yet when these conditions are peculiarly favorable, condylomas can invade normal tissues, and be highly destructive.<sup>8</sup> In numerous reported instances (14) this has happened when the growth was pent beneath a phimotic foreskin, and

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<sup>7</sup> At this writing the cancers of D. R. 2-38 are, on the average, less than one-fourth their size at one time.

<sup>8</sup> We are indebted to Dr. Marion B. Sulzberger for bringing these cases to our notice.

there was inflammation due to bacteria. Then it has invaded and perforated the foreskin, preceded by cellulitis, and burrowed through the *corpora cavernosa* and *spongiosum*, causing urethral fistulae. Amputation of the penis has proved the only safe course. In some cases the growth has remained a condyloma histologically (*carcinomähnlich Condyloma*, "the pathologists find nothing, yet it is cancer" (15)), and again it has given more or less outspoken morphological signs of malignant change, or has undergone the transformation to a typical squamous cell carcinoma yielding metastases. Often biopsy has shown a condyloma but the recurrence has been carcinomatous.

This progression to cancer of a notably benign growth caused by a virus dependent for its action on favoring local conditions, involves changes of far greater scope than are required of the Shope rabbit papilloma when becoming malignant. Yet for the effectiveness of even so drastic a carcinogenic agent as the Shope virus, numerous conditions must be right. The virus must find its way into animals of the proper species, into favorable individuals, into especially susceptible skin (such as responds well to stimulation with Scharlach R), into an association with the epidermis (pigmented being very favorable), and even then it may not set carcinosis in train unless local factors contribute. Malignancy is the outcome of numerous concurring influences, as holds true of cancer generally (5).

#### SUMMARY

The papillomas induced in domestic rabbits with virus procured from cottontails undergo progressive changes in the direction of malignancy when they grow vigorously. From the beginning they exhibit the traits whereby tumors are characterized, and they have malignant potentialities. In seven animals of a group of ten carrying papillomas for more than 200 days, cancer has developed, and in an eighth a tumor of problematic malignancy has arisen. One of the remaining two rabbits died early in the cancer period, and the papillomas of the other eventually retrogressed. Ten cottontails with induced growths of much longer duration have not developed cancer.

The malignant tumors have all been acanthomatous in type, and have arisen directly from the papillomas by graded, continuous alterations. These have often gone further after malignancy has been attained, and have eventuated in great anaplasia. Metastasis has

been frequent, and transplantation to another host has proved successful. Individual growths have occurred expressive of each stage of the transformation to cancer, as if through a stabilization at this stage; yet despite the variety thus afforded, the tumors must all be looked upon as the consequence of alterations in cells of a single sort, namely epidermal cells affected by the virus, and the alterations themselves have taken a single direction. In the morphology of many of the cancers the influence of the virus is still manifest.

The better the papilloma grew, the more likely was cancer to occur, and the greater was the tendency to multiple tumors. In the most favorable rabbits malignant changes took place at numerous locations in the papillomatous tissue, and were imminent at many others. Intercurrent factors had much to do with determining frank carcinosis; and when the tendency to it was not marked their influence sometimes seemed crucial.

Analogous instances of a graded alteration from papilloma to cancer are frequent in human pathology. The virus that gives rise to the rabbit papillomas must be looked upon as the primary cause of the cancers developing therefrom. Whether it is their proximate cause has yet to be determined.

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## EXPLANATION OF PLATES

All of the sections were stained with eosin and methylene blue. S indicates the side on which the skin surface was.

## PLATE 19

FIG. 1. Ulcerated carcinoma where was once a papilloma like those still present. The arrows indicate the direction of the subsequent biopsy cuts. *D. R. 2-38*: 214 days.  $\times 5/11$ .

FIG. 2. Cross-section of the cancer of Fig. 1. 214 days.  $\times 6\frac{3}{4}$ .

FIG. 3. Edge of a metastasis in an axillary node removed on the 237th day.

FIG. 4. The carcinoma of Fig. 1, photographed 78 days later. It has undermined and involved the neighboring papillomas, and coalesced with a cancer arising in one of these. The others are in a precancerous state, much less pigmented than previously and with thickened, deep bases. A second axillary metastasis has developed (arrow).  $\times 7/11$ .

FIG. 5. Invasive, cystic growth deriving from a papilloma. It has extended out under the neighboring skin. *D. R. 2-52*: 344 days. Fig. 22 shows the gross specimen. The present figure has been reversed in its relation to this. (See also Figs. 14 and 15.)  $\times 4\frac{1}{2}$ .

## PLATE 20

FIG. 6. Ulcerating cancer that has replaced one papillomatous mass and is encroaching upon another. The arrows A and B point to the regions of successive, later biopsies. *D. R. 2-39*: 211 days.  $\times 10/11$ .

FIG. 7. Cancer (A) extending from beneath a fleshy papilloma. It has raised the skin slightly. The axillary metastasis (B) attracted attention 21 days before the primary tumor was palpable. Some of the papillomas are fleshy and thick-based, whereas others have not undergone these precancerous alterations. *D. R. 2-53*: 329 days.  $\times 5/11$ .

FIG. 8. Section through the edge (A) of the ulcer of Fig. 6, with adjacent skin. The growth is a malignant papilloma, breaking up into squamous cell carcinoma which has invaded the voluntary muscle. 212 days.  $\times 7\frac{1}{2}$ .

FIG. 9. Section from the tip of the subcutaneous extension (B) of the same tumor. Transition forms from papilloma to squamous cell carcinoma are again found. 230 days.  $\times 31$ .

FIG. 10. Part of a nodule removed from the leg muscles 12 days after implantation of the material of Fig. 9. The tumor is now almost entirely of squamous cell type.  $\times 31$ .

## PLATE 21

FIG. 11. Retroperitoneal metastasis from the tumor of Figs. 8, 9, 10, invading the wall of the aorta (upper part of figure). The growth is far more anaplastic than previously. 298 days.  $\times 130$ .

FIG. 12. Early extension of a Shope papilloma into the corium. *D. R. 1-92*: 47 days.  $\times 90$ .

FIG. 13. Later irregularity and extension downwards of another papilloma, with pearl formation. *D. R. 1-55*: 72 days.  $\times 8$ .

FIG. 14. Malignant (?), cystic extension of a papilloma into the subcutaneous tissue (see also Figs. 5, 17, and 22). *D. R. 2-35*: 286 days.  $\times 5$ .

FIG. 15. Early malignant changes within a papilloma. The irregularity of the uninvolved portion to right, should be noted. From the same extensive, papillomatous mass as Fig. 14.  $\times 5$ .

## PLATE 22

FIG. 16. Papilloma that has undergone precancerous changes. The high peaks have been replaced by flaky material, and the base of the growth is fleshy, with a raised, tense border. A large, subepidermal pearl has recently appeared (arrow) outside it. *D. R. 2-39*: 238 days.  $\times 7/11$ .

FIG. 17. Localized downgrowth from the middle of the base of a long-established Shope papilloma. *D. R. 2-52*: 210 days.  $\times 6\frac{1}{2}$ .

FIG. 18. Fungoid carcinomas which have replaced 3 papillomas. They have been gnawed, whereas the papillomatous masses to either side are intact. The arrow indicates where a biopsy was to be done. *D. R. 2-52*: 175 days.  $\times 7/11$ .

FIG. 19. Section through one side of the growth designated in Fig. 18, with the adjacent skin. It is a malignant papilloma. 176 days.  $\times 14$ .

FIG. 20. Malignant, non-pigmented papilloma extending under an ordinary papilloma (arrow) that is heavily pigmented. *D. R. 2-35*: 286 days.  $\times 7\frac{1}{2}$ .

## PLATE 23

FIG. 21. Section through a superficial part of the fungating tumor shown in Fig. 28. *D. R. 2-48*: 385 days.  $\times 8$ .

FIG. 22. Section through the mass that eventually took the place of all of the growths shown in Fig. 18. Some of the Shope papillomas still persisted as such. The new tumors that had arisen from them, or invaded them and coalesced, were mostly malignant papillomas. The brackets indicate part of a cystic tumor, which is shown in Fig. 5. Natural size.

FIG. 23. Squamous cell carcinoma deriving directly from the base of a Shope papilloma. (From another part of the papillomatous mass furnishing Figs. 14, 15, and 20.)  $\times 35$ .



FIG. 24. Metastasis in an axillary lymph node of a squamous cell carcinoma with cystic tendencies. *D. R. 2-05*: 348 days.  $\times 6\frac{1}{2}$ .

FIG. 25. Malignancy developing in the wall of a pearl under an old papilloma. (See also Fig. 26.) *D. R. 2-35*: 233 days.  $\times 7\frac{1}{2}$ .

PLATE 24

FIG. 26. The region indicated with an arrow in Fig 25. Bacterial infection and local inflammation are present where the epithelium has invaded the connective tissue.  $\times 140$ .

FIG. 27. Part of a tumor resulting from transplantation to another rabbit of the cancer of *D. R. 2-39*, which is shown in Figs. 8, 9, and 10.  $\times 31$ .

FIG. 28. Fungating tumor of questionable malignancy, which has almost entirely replaced a papillomatous mass. Some of the latter persists along the edges as dry, sooty peaks. (See Fig. 21.) *D. R. 2-48*: 384 days.  $\times 5/8$ .

FIG. 29. Cancers appearing as discoid growths in the midst of confluent, papillomatous expanses. *D. R. 2-35*: 244 days.  $\times 5/16$ .

FIG. 30. One of the cancers of Fig. 29. 286 days.  $\times 190$ .

FIG. 31. Lung metastasis in the animal of Fig. 29, with the morphology of Fig. 30. 286 days.  $\times 190$ .



Photographed by Louis Schmidt and Joseph B. Haulenbeck

(Rous and Beard Virus-induced rabbit papillomas (Shope))

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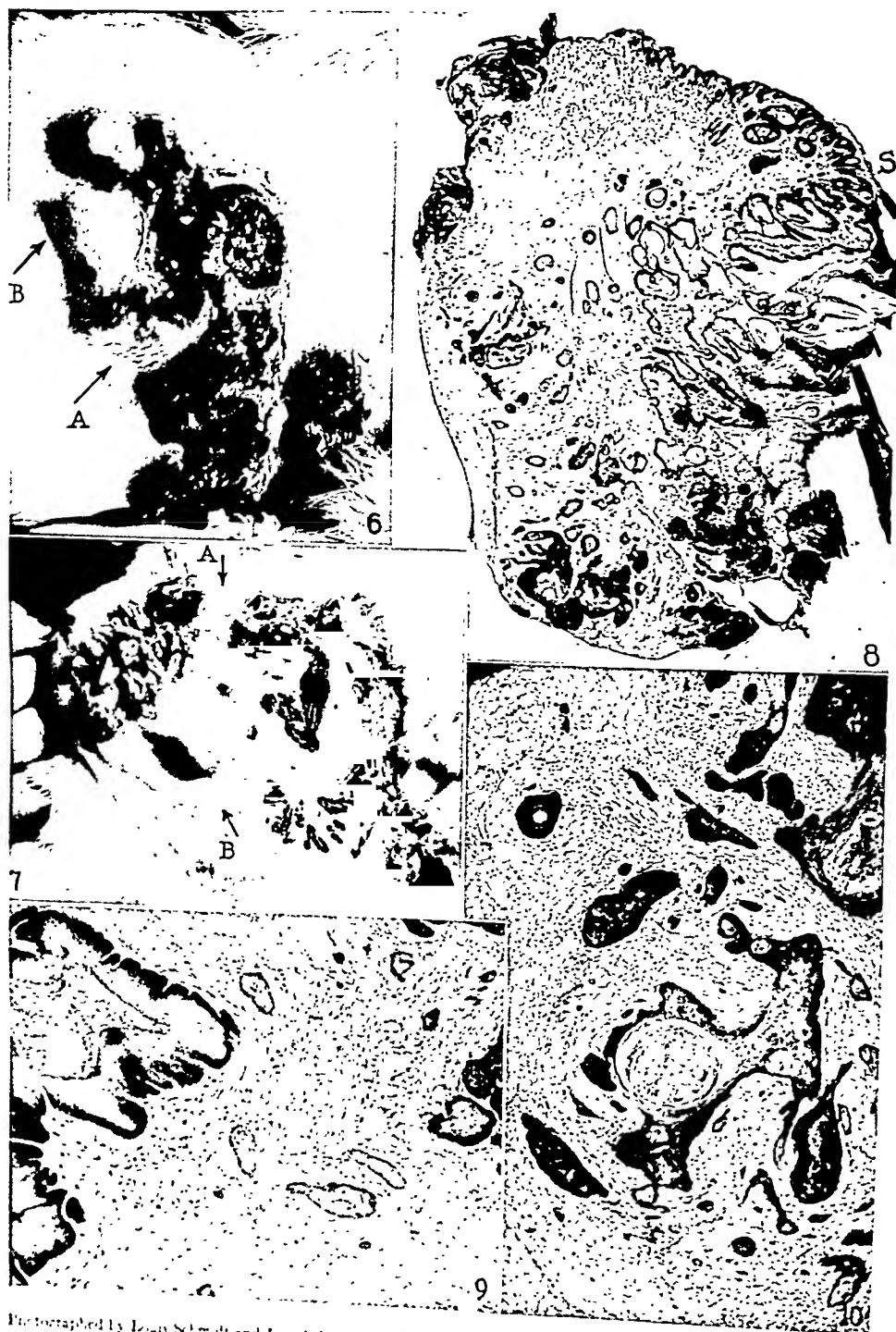
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Photographed by Louis Schmidt and Joseph B. Haulenbeck

(Rous and Beard: Virus-induced rabbit papillomas (Shope))





Photomicrographs by Louis Schmitt and Joseph B. Haulenbeck

(Rous and Beard: Virus-induced rabbit papillomas (Shope))





Photomicrographs by Louis Schmidt and Joseph B. Haulenbeck

Orizaba and Bear I. Virus in fetal rat pituitary (Stegmann)



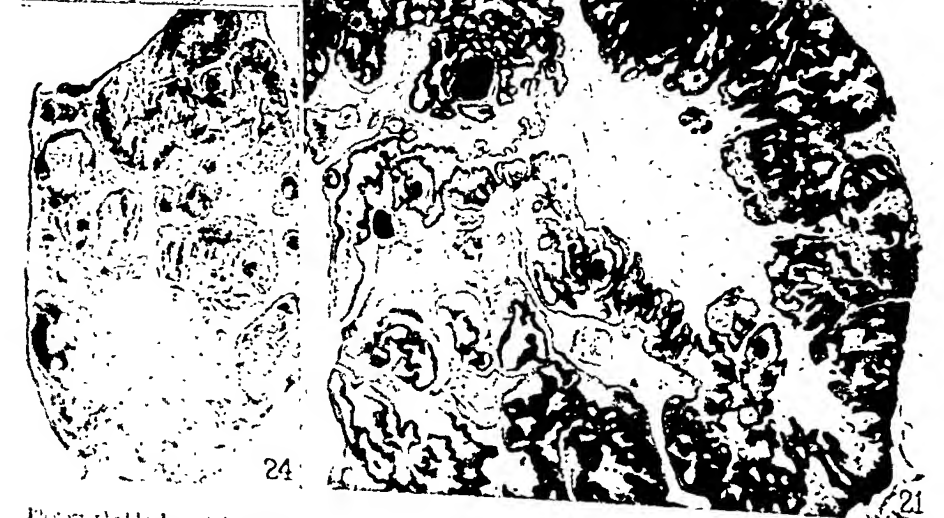
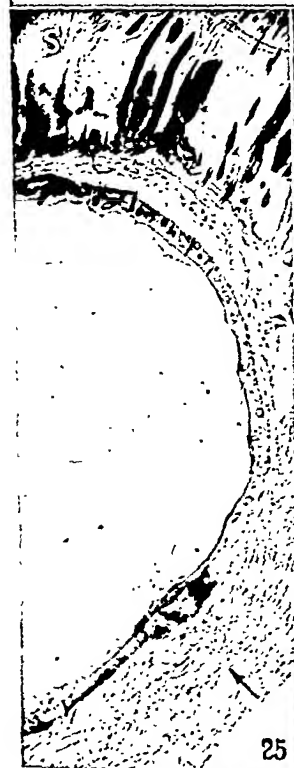




Photographs by Louis Seemilt and Joseph B. Haulenbeck

(Rous and Beard: Virus-induced rabbit papillomas (Sten.)









1. Courtesy of L. S. Schram and J. Joseph B. Haulerbeck

(Rous and Dear, Virus in feral rabbit papillomas (Shope))



# JENNERIAN PROPHYLAXIS BY MEANS OF IN- TRADERMAL INJECTIONS OF CULTURE VACCINE VIRUS

BY THOMAS M. RIVERS, M.D., AND S. M. WARD

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PLATE 25

(Received for publication, June 28, 1935)

The successful cultivation of vaccine virus in a medium consisting of minced chick embryo tissue suspended in Tyrode's solution and the use of this virus by means of dermal inoculation for Jennerian prophylaxis in man have been described in previous communications (1, 2). A continuation of investigations of culture vaccine virus has rendered possible at this time a statement regarding the desirability of its intradermal use in man and a presentation of facts pertaining to its preservation and application.

In an earlier report (2) the fact that the titer of our vaccine gradually diminished during the course of successive passages in the medium used was recorded. At that time there was also described a strain of culture virus, designated as the second revived strain, which maintained a constant titer and appeared to have become adapted to *in vitro* cultivation. This second revived strain has now been carried through 130 successive passages in chick embryo tissue and Tyrode's solution during a period of 3 years and still regularly yields when injected intradermally into rabbits typical mild vaccinal lesions. It is assumed, therefore, that serial passage in cultures may be continued much longer, or that by reversion to earlier cultures preserved at low temperatures the necessity of further rejuvenation by passage through animals will seldom arise.

## *Intradermal Vaccination against Smallpox*

Since the cultivation of vaccine virus *in vitro* has made available an active agent free from bacteria and now sufficiently mild in its



action to be injected with safety into the skin of human beings, we believe that intradermal vaccination deserves serious consideration as a means of preventing smallpox. The procedure is quickly and easily executed and the amount of material inoculated can be controlled accurately. The resultant reactions are characterized by erythema and induration without the formation of an open sore and a disfiguring scar. There is, therefore, little opportunity for the occurrence of secondary infections. The absence of a vaccinal scar is not an undesirable feature since the presence of such a scar is no assurance of immunity to smallpox. Furthermore, the reactions produced by intradermal injections of vaccine virus in individuals who have been previously vaccinated are characteristic and more susceptible of interpretation in terms of resistance to infection than are the reactions that follow dermal revaccination.

### *Method of Intradermal Inoculation*

Routine cultures of vaccine virus after being ground in a mortar without an abrasive are tested for the presence of ordinary bacteria. Bacteria-free emulsions are then mixed with equal amounts of sterile glycerol and stored at a temperature several degrees below 0°C. With such material most of our intradermal vaccinations and revaccinations have been conducted in the following manner. 0.1 cc. of glycerolated culture virus diluted 5-10 times with sterile salt solution is injected into the skin of the upper arm or thigh by means of a tuberculin syringe fitted with a 27 gauge needle in a manner similar to that used in making an intradermal tuberculin or Schick test. After the injection has been completed it is essential to cleanse the needle wound thoroughly with alcohol in order to prevent the formation of a vesicle on the surface of the skin. No dressing or special care is indicated; *i.e.*, the arm or thigh can be freely bathed throughout the evolution of the intradermal lesion.

### *Results of Primary Intradermal Vaccinations*

In the clinic at the Hospital of The Rockefeller Institute there is no opportunity of vaccinating a large number of people. Nevertheless, from time to time we are requested to make primary vaccinations, and during the last year and a half 29 infants and children have received the culture virus intradermally.

When an individual who has never had a successful vaccination receives the culture virus intradermally, nothing abnormal is noted at the point of inoculation until 4 to 9 days later. Then a small red papule appears which gradually becomes

larger until the area of induration and erythema is 2 or 3 cm. in diameter (Fig. 1). At the height of the reaction, which is usually 4 to 6 days after its initiation, a secondary zone of erythema, less intense than the primary, makes its appearance around the lesion, remains for a day or two, and then rapidly disappears. The induration gradually becomes less marked and as a rule is no longer discernible 3 weeks after the appearance of the lesion. If the inoculation is made properly there is no vesicle formation to leave a scar.

The 29 infants and children received one intradermal inoculation of the second revived strain of culture vaccine virus (42<sub>2</sub>-87<sub>2</sub> culture generations were used) that had been stored with glycerol 6 days to 6 months. All of them reacted with primary takes.

19 of them were under observation to such an extent that we could determine the time of appearance of the lesions; 2 appeared on the 4th day after inoculation, 2 on the 5th day, 3 on the 6th day, 5 on the 7th day, 2 on the 8th day, 2 on the 9th day, and 1 each on the 10th, 13th, and 17th days. None of the children seemed sick as a result of the vaccinations. While most of them showed an increase in the size of the axillary lymph nodes, only 2 had fever, and in them the elevation of temperature lasted only one day and went no higher than 101°F. One of the subjects that responded with a primary take had been unsuccessfully vaccinated on 3 occasions previously in which calf lymph and the scratch method of inoculation had been employed.

It has not been possible to make tests for immunity to calf lymph in all of the individuals that showed primary takes resulting from intradermal inoculation of culture virus. In 7 instances, however, such tests have been made. 6 infants revaccinated dermally with the New York City Board of Health calf lymph, 13 days, 16 days, 17 days, 19 days, 20 days, and 6 months, respectively, after a primary intradermal vaccinal lesion responded with immune reactions, while 1 infant revaccinated after a lapse of 7 months responded with an accelerated take.

#### *Results of Revaccination by the Intradermal Method*

Revaccinations by means of calf lymph applied dermally result in immediate or immune reactions, or in accelerated takes. Most investigators admit, however, that it is difficult to differentiate an immune reaction from no reaction, because trauma alone incident to a dermal vaccination induces a certain amount of reaction that leads to confusion. In view of this fact, we attempted to ascertain whether

intradermal injections of culture virus in individuals previously successfully vaccinated would yield distinct and unmistakable immune reactions or accelerated takes. If such reactions regularly occur, then the absence of vaccinal scars would not be a valid reason for opposing intradermal vaccination, because within a short time one could determine whether an individual is immune to vaccinia or not.

33 individuals, 1 to 65 years of age, who had been successfully vaccinated one or more times with calf lymph, received intradermal injections of culture vaccine virus similar to those used for primary inoculations. All of them, with the exception of 2, responded with reactions comparable either to the immune reactions or to the accelerated takes that follow dermal revaccinations. The earliest reaction to occur appeared within 6 hours after inoculation, while others did not become evident until 2 or 3 days later. Those that appeared rapidly consisted of small red papules accompanied by itching and disappeared in a day or two. The ones that developed more slowly were larger than the others, endured for 3 or 4 days, and gradually disappeared to be followed by a slight desquamation of the involved skin.

The fact that no reactions were observed until from 4 to 17 days after primary intradermal inoculations of the culture virus led us to believe that the immediate and accelerated reactions encountered in revaccinated people were specific for vaccinia. To test the specificity of the reactions further, uninoculated media incubated and handled in a manner similar to that of the cultures were in many instances injected as a control when the revaccinations were made. None of the control injections produced demonstrable reactions. At this time it is of interest to record the fact that cultures of vaccine virus passed through filters which retain all of the infectious agent contain a soluble antigen that is innocuous for the skin of unvaccinated people but capable of producing a reaction in the skin of individuals immune to vaccinia, while filtrates of uninoculated media cause no obvious changes in the skin of either group.

### *Preservation of Culture Virus*

Culture vaccine virus stored in 50 per cent glycerol retains its activity for several years and can be used for vaccination or the initiation of cultures. Such virus, however, does not remain active in the absence of refrigeration, a feature that has interfered with the dis-

tribution of the active agent by mail. In view of the fact that many viruses desiccated in a frozen state retain their activity under conditions ordinarily deleterious, we have attempted to obtain preparations of desiccated culture vaccine virus that are suitable for human use and retain their activity for considerable periods of time in the absence of refrigeration.

Some results obtained with desiccated culture vaccine virus have already been described (2). However, experience has shown that when such virus is dried in small amounts in glass tubes difficulty is encountered in removing the active agent from the containers. The virus apparently adheres to the walls of the tubes to such an extent that when fluid is added and the material from each of several tubes of the same batch is titrated in the skin of the same rabbit uniform results are not realized. Therefore, it became necessary to add to the culture virus some sterile innocuous substance that would act as a protective agent, add bulk to the dried preparations, and go into solution or become suspended with ease upon the addition of fluid. Egg albumen was the material investigated first.

### *Preservation of Culture Vaccine Virus in the Presence of Egg Albumen*

Mixtures of culture virus and egg albumen were desiccated and handled in the following manner.

3 parts of culture virus, ground in a mortar without an abrasive until the particles of tissue are finely dispersed, are thoroughly mixed with 1 part of sterile egg albumen. The mixture is then distributed in 0.25 cc. amounts into small sterile test tubes, frozen rapidly by means of solid  $\text{CO}_2$  in alcohol, and placed in a refrigerating box at  $-4^\circ\text{C}$ . in the presence of  $\text{CaCl}_2$ . Air is evacuated from the box by means of a Cenco-Hyvac pump, and throughout the period of desiccation the pressure in the box is maintained at approximately 1-2 mm. of mercury. Drying of the virus and egg albumen occurs rapidly. The tubes are usually allowed to remain in the box for 24 hours after which they are removed and placed in a desiccator at room temperature. When the tubes have returned to the temperature of the room they are removed from the desiccator and quickly sealed in a hot flame. The dried material appears as white foam-like pellets which can be freed with ease from the sides of the tubes and pulverized. It is readily miscible in water or saline solution.

A number of samples of culture virus have been dried with egg albumen and have been shown to retain their activity for considerable periods of time when held at room temperature. One experiment, summarized in Table I, showed by intradermal tests in rabbits that the virus kept under such conditions for  $3\frac{1}{2}$  months was still active.

Desiccated mixtures of culture virus and egg albumen may be used for dermal vaccination of human beings.

A tube is opened aseptically, the dried material is pulverized in the container, a small amount of the powder is taken up on the end of a sterile wooden applicator and is rubbed on a scarified area of skin of the upper arm or thigh. The moist serous exudate resulting from the scarification dissolves the powder sufficiently

TABLE I  
*Record of Intradermal Titrations in Rabbits of Vaccine Virus, Culture 662, Desiccated with Egg Albumen and Stored at Room Temperature*

| Time of titration                  |   |   | Titer of virus                      |
|------------------------------------|---|---|-------------------------------------|
| Before desiccation.....            |   |   | 10 <sup>-5</sup>                    |
| Immediately after desiccation..... |   |   | 10 <sup>-5</sup> ; 10 <sup>-6</sup> |
| 5 days                             | " | " | 10 <sup>-5</sup>                    |
| 16 days                            | " | " | 10 <sup>-5</sup>                    |
| 1 mo.                              | " | " | 10 <sup>-6</sup>                    |
| 2 mos.                             | " | " | 10 <sup>-5</sup>                    |
| 3½ mos.                            | " | " | 10 <sup>-4</sup>                    |
|                                    |   |   | 10 <sup>-3</sup>                    |

to cause it to adhere to the skin. The evolution of the vaccinal lesions induced in this way is normal and similar to that caused by moderately potent commercial vaccine virus.

Eleven of 12 children who received dermal applications of the virus dried with egg albumen responded with typical primary takes. One of them was sensitive to the albumen and a large urticarial lesion developed almost immediately at the site of inoculation. This disappeared, however, and the evolution of the vaccinal lesion was entirely normal.

Egg white is a highly antigenic substance. In view of this fact, although the use of mixtures of virus and albumen for dermal vaccination is not attended by harmful results even in individuals already sensitive to egg white, one hesitates to advocate the use of such

preparations for intradermal vaccination. Inasmuch as we were particularly interested in developing a method of preserving the culture virus for intradermal use in human beings, we sought for harmless non-antigenic substances that would protect the active agent during the processes of drying and resuspension. Purified gum acacia has been found to act satisfactorily.

*Preservation of Culture Vaccine Virus in the Presence of Purified  
Gum Acacia*

A 30 per cent solution of gum acacia, purified for intravenous administration, was obtained from Eli Lilly and Co. Intradermal injections of this material in concentrations as high as 7.5 per cent caused no perceptible reactions in human beings. Mixtures of culture virus and acacia were desiccated with ease, and when dry they went back into solution readily upon the addition of water or saline solution. It appeared that we had found an ideal means of preserving the virus with the exception that the apparatus used for freezing and drying necessitated the exposure of the desiccated material to atmospheric pressure and moisture before the containers were sealed. We were aware, therefore, that a method of desiccation which would permit the containers of the dried product to be sealed while under a vacuum would be an improvement. Recently, Mudd and his associates (3, 4) have described an apparatus designed for this purpose, and we have adapted it to the preservation of our culture virus.

The Mudd apparatus (Fig. 2) used by us consists of a cylindrical glass bulb to which on one side near the top is fused in a horizontal position a long hollow glass side arm or manifold with 24 apertures. On the opposite side, the bulb is attached by a short side arm and rubber stopper to a trap which in turn is connected by pressure tubing to a Cenco-Hyvac pump. The bulb and trap are placed in a mixture of solid CO<sub>2</sub> and alcohol in a thermos jug. The material to be dried is frozen in pyrex glass containers which are then suspended from the apertures of the side arm or manifold by means of units consisting of rubber tubing, glass tubing, and a hollow rubber cork. Then the pressure in the apparatus is reduced rapidly to a low level, the material to be dried remains frozen, and the moisture drawn from the containers condenses in the large bulb immersed in the freezing mixture. The containers of the dried virus are sealed, while the vacuum pump is still running, by means of a torch equipped with 2 tips opposing each other and supplied with a mixture of oxygen and gas.

The method which we now use for the preservation of culture vaccine virus to be employed for vaccination of human beings is as follows.

1 part of a sterile 30 per cent solution of gum acacia is mixed with 11 parts of culture virus which has been ground in a mortar without an abrasive until the particles of tissue are finely dispersed. 0.4 or 0.8 cc. of the mixture are placed in each of a number of ampoules. The ampoules, blown from pyrex glass tubing with an internal diameter of approximately 5 mm., have necks about 8.5 cm. in length, flat bottoms, and a capacity of at least 2 cc. The virus is placed in the ampoules by means of a syringe with a long needle, after which the neck of each ampoule is inserted into one end of a short piece of pressure gum rubber tubing the other end of which receives an arm of a short piece of glass tubing bent to form a right angle. The other arm of the glass tube passes through a gum rubber stopper that fits an opening in the manifold of the drying apparatus. Each time before they are used, the bent glass tubes, with one arm pushed through its hollow rubber stopper and closed by a gauze plug and the other arm inserted into its short piece of rubber tubing, are wrapped separately in paper and sterilized in an autoclave. After 24 units consisting of ampoules, rubber tubing, glass tubing, and stoppers have been assembled in the manner described the contents of the ampoules are frozen by immersion in a mixture of solid  $\text{CO}_2$  and alcohol. Then the units are rapidly connected with the drying apparatus by insertion of the rubber stopper of each unit into an aperture of the manifold after removal of the gauze plug from the end of the glass tube. The vacuum pump is started immediately, and within a few minutes the outer surface of the ampoules becomes covered with frost which gradually evaporates as desiccation proceeds. Desiccation is completed within 5 or 6 hours, and the necks of the ampoules are then sealed by means of an oxygen torch while the vacuum pump is still running.

When the dried material is desired for use an ampoule is opened and 2 cc. of sterile water or saline solution is added by means of a syringe and needle. In this manner a 1 to 2.5 or a 1 to 5 dilution of the virus is effected depending on whether 0.8 cc. or 0.4 cc. of virus have been placed in the ampoules before desiccation. The 1 to 2.5 or 1 to 5 dilutions of the virus may be used for intradermal vaccination of human beings, or by means of further dilutions they may be used for intradermal titrations in rabbits.

In view of previous experiences we have assumed that the virus dried in the presence of acacia will remain active for long periods of time when stored at low temperatures. However, we wanted to know definitely whether it would retain its activity under conditions encountered during transportation requiring several days or weeks. Consequently, we have tested numerous batches of the dried culture

|                           | N32 | N33 | N34 | N35 |
|---------------------------|-----|-----|-----|-----|
| 1. Time of storage, days  | 10  | 10  | 10  | 10  |
| 2. Percent survival       | 100 | 100 | 100 | 100 |
| 3. Time of storage, days  | 10  | 10  | 10  | 10  |
| 4. Percent survival       | 100 | 100 | 100 | 100 |
| 5. Time of storage, days  | 10  | 10  | 10  | 10  |
| 6. Percent survival       | 100 | 100 | 100 | 100 |
| 7. Time of storage, days  | 10  | 10  | 10  | 10  |
| 8. Percent survival       | 100 | 100 | 100 | 100 |
| 9. Time of storage, days  | 10  | 10  | 10  | 10  |
| 10. Percent survival      | 100 | 100 | 100 | 100 |
| 11. Time of storage, days | 10  | 10  | 10  | 10  |
| 12. Percent survival      | 100 | 100 | 100 | 100 |
| 13. Time of storage, days | 10  | 10  | 10  | 10  |
| 14. Percent survival      | 100 | 100 | 100 | 100 |
| 15. Time of storage, days | 10  | 10  | 10  | 10  |
| 16. Percent survival      | 100 | 100 | 100 | 100 |
| 17. Time of storage, days | 10  | 10  | 10  | 10  |
| 18. Percent survival      | 100 | 100 | 100 | 100 |
| 19. Time of storage, days | 10  | 10  | 10  | 10  |
| 20. Percent survival      | 100 | 100 | 100 | 100 |
| 21. Time of storage, days | 10  | 10  | 10  | 10  |
| 22. Percent survival      | 100 | 100 | 100 | 100 |
| 23. Time of storage, days | 10  | 10  | 10  | 10  |
| 24. Percent survival      | 100 | 100 | 100 | 100 |
| 25. Time of storage, days | 10  | 10  | 10  | 10  |
| 26. Percent survival      | 100 | 100 | 100 | 100 |
| 27. Time of storage, days | 10  | 10  | 10  | 10  |
| 28. Percent survival      | 100 | 100 | 100 | 100 |
| 29. Time of storage, days | 10  | 10  | 10  | 10  |
| 30. Percent survival      | 100 | 100 | 100 | 100 |
| 31. Time of storage, days | 10  | 10  | 10  | 10  |
| 32. Percent survival      | 100 | 100 | 100 | 100 |
| 33. Time of storage, days | 10  | 10  | 10  | 10  |
| 34. Percent survival      | 100 | 100 | 100 | 100 |
| 35. Time of storage, days | 10  | 10  | 10  | 10  |
| 36. Percent survival      | 100 | 100 | 100 | 100 |
| 37. Time of storage, days | 10  | 10  | 10  | 10  |
| 38. Percent survival      | 100 | 100 | 100 | 100 |
| 39. Time of storage, days | 10  | 10  | 10  | 10  |
| 40. Percent survival      | 100 | 100 | 100 | 100 |
| 41. Time of storage, days | 10  | 10  | 10  | 10  |
| 42. Percent survival      | 100 | 100 | 100 | 100 |
| 43. Time of storage, days | 10  | 10  | 10  | 10  |
| 44. Percent survival      | 100 | 100 | 100 | 100 |
| 45. Time of storage, days | 10  | 10  | 10  | 10  |
| 46. Percent survival      | 100 | 100 | 100 | 100 |
| 47. Time of storage, days | 10  | 10  | 10  | 10  |
| 48. Percent survival      | 100 | 100 | 100 | 100 |
| 49. Time of storage, days | 10  | 10  | 10  | 10  |
| 50. Percent survival      | 100 | 100 | 100 | 100 |
| 51. Time of storage, days | 10  | 10  | 10  | 10  |
| 52. Percent survival      | 100 | 100 | 100 | 100 |
| 53. Time of storage, days | 10  | 10  | 10  | 10  |
| 54. Percent survival      | 100 | 100 | 100 | 100 |
| 55. Time of storage, days | 10  | 10  | 10  | 10  |
| 56. Percent survival      | 100 | 100 | 100 | 100 |
| 57. Time of storage, days | 10  | 10  | 10  | 10  |
| 58. Percent survival      | 100 | 100 | 100 | 100 |
| 59. Time of storage, days | 10  | 10  | 10  | 10  |
| 60. Percent survival      | 100 | 100 | 100 | 100 |
| 61. Time of storage, days | 10  | 10  | 10  | 10  |
| 62. Percent survival      | 100 | 100 | 100 | 100 |
| 63. Time of storage, days | 10  | 10  | 10  | 10  |
| 64. Percent survival      | 100 | 100 | 100 | 100 |
| 65. Time of storage, days | 10  | 10  | 10  | 10  |
| 66. Percent survival      | 100 | 100 | 100 | 100 |
| 67. Time of storage, days | 10  | 10  | 10  | 10  |
| 68. Percent survival      | 100 | 100 | 100 | 100 |
| 69. Time of storage, days | 10  | 10  | 10  | 10  |
| 70. Percent survival      | 100 | 100 | 100 | 100 |
| 71. Time of storage, days | 10  | 10  | 10  | 10  |
| 72. Percent survival      | 100 | 100 | 100 | 100 |
| 73. Time of storage, days | 10  | 10  | 10  | 10  |
| 74. Percent survival      | 100 | 100 | 100 | 100 |
| 75. Time of storage, days | 10  | 10  | 10  | 10  |
| 76. Percent survival      | 100 | 100 | 100 | 100 |
| 77. Time of storage, days | 10  | 10  | 10  | 10  |
| 78. Percent survival      | 100 | 100 | 100 | 100 |
| 79. Time of storage, days | 10  | 10  | 10  | 10  |
| 80. Percent survival      | 100 | 100 | 100 | 100 |
| 81. Time of storage, days | 10  | 10  | 10  | 10  |
| 82. Percent survival      | 100 | 100 | 100 | 100 |
| 83. Time of storage, days | 10  | 10  | 10  | 10  |
| 84. Percent survival      | 100 | 100 | 100 |     |

[illegible]



The method which we now use for the preservation of culture vaccine virus to be employed for vaccination of human beings is as follows.

1 part of a sterile 30 per cent solution of gum acacia is mixed with 11 parts of culture virus which has been ground in a mortar without an abrasive until the particles of tissue are finely dispersed. 0.4 or 0.8 cc. of the mixture are placed in each of a number of ampoules. The ampoules, blown from pyrex glass tubing with an internal diameter of approximately 5 mm., have necks about 8.5 cm. in length, flat bottoms, and a capacity of at least 2 cc. The virus is placed in the ampoules by means of a syringe with a long needle, after which the neck of each ampoule is inserted into one end of a short piece of pressure gum rubber tubing the other end of which receives an arm of a short piece of glass tubing bent to form a right angle. The other arm of the glass tube passes through a gum rubber stopper that fits an opening in the manifold of the drying apparatus. Each time before they are used, the bent glass tubes, with one arm pushed through its hollow rubber stopper and closed by a gauze plug and the other arm inserted into its short piece of rubber tubing, are wrapped separately in paper and sterilized in an autoclave. After 24 units consisting of ampoules, rubber tubing, glass tubing, and stoppers have been assembled in the manner described the contents of the ampoules are frozen by immersion in a mixture of solid CO<sub>2</sub> and alcohol. Then the units are rapidly connected with the drying apparatus by insertion of the rubber stopper of each unit into an aperture of the manifold after removal of the gauze plug from the end of the glass tube. The vacuum pump is started immediately, and within a few minutes the outer surface of the ampoules becomes covered with frost which gradually evaporates as desiccation proceeds. Desiccation is completed within 5 or 6 hours, and the necks of the ampoules are then sealed by means of an oxygen torch while the vacuum pump is still running.

When the dried material is desired for use an ampoule is opened and 2 cc. of sterile water or saline solution is added by means of a syringe and needle. In this manner a 1 to 2.5 or a 1 to 5 dilution of the virus is effected depending on whether 0.8 cc. or 0.4 cc. of virus have been placed in the ampoules before desiccation. The 1 to 2.5 or 1 to 5 dilutions of the virus may be used for intradermal vaccination of human beings, or by means of further dilutions they may be used for intradermal titrations in rabbits.

In view of previous experiences we have assumed that the virus dried in the presence of acacia will remain active for long periods of time when stored at low temperatures. However, we wanted to know definitely whether it would retain its activity under conditions encountered during transportation requiring several days or weeks. Consequently, we have tested numerous batches of the dried culture



virus that had been stored at 37°C. for 2 and 4 weeks. From the results of the experiments, summarized in Table II, it is obvious that the dried virus retains its activity remarkably well under such conditions.

We have been able to vaccinate and revaccinate only a few people with mixtures of virus and acacia. In every instance, however, the intradermal inoculation of the material resulted in primary reactions, accelerated takes, or immune responses.

Inasmuch as we are in no position to vaccinate a large number of people, the dried mixture of culture virus and acacia is being supplied to a group of physicians who will determine the rapidity of appearance and duration of immunity resulting from its intradermal use. Already more than a thousand individuals have been vaccinated and in the course of time reports concerning the value of intradermal vaccination with the culture virus will appear in the literature.

#### DISCUSSION

Intradermal vaccination of human beings against smallpox has been tried by a number of investigators and Roberts (5) has recently reviewed the literature on the subject. It should be clearly understood that we are not advocating the intradermal use of ordinary calf lymph or cultures of vaccine virus recently initiated from calf lymph or other sources, because such materials (1, 5) used intradermally may result in more severe constitutional disturbances and damages to the tissues at the site of inoculation than those caused by their dermal application. Although our culture virus came originally from the New York City Board of Health calf lymph it is free from ordinary bacteria and has now been propagated for such a length of time *in vitro* that mild reactions are regularly produced when it is injected intradermally in rabbits and human beings.

Our second revived strain of culture virus can be used with safety for intradermal vaccination of human beings, and when handled properly it induces a high percentage of takes which are followed within a reasonable length of time by an immunity to a standard dermal strain of vaccine virus. Consequently, we are convinced that the intradermal use of culture virus should be seriously considered as a method to be employed more extensively for protection against smallpox.

Knowledge concerning the duration of immunity produced by the culture virus is not available yet. Nevertheless, this will be obtained gradually. When the facts regarding the matter are presented, investigators and physicians should not be surprised to find that a certain number of successfully vaccinated individuals will have lost their immunity within a period of a year. At least that is the case with people who have been successfully vaccinated for the first time with calf lymph. For instance, Kitasato (6) found that 13.3 per cent of people successfully vaccinated with calf lymph were again susceptible a year later. Donnally and Nicholson (7), using a standard calf lymph, vaccinated 500 infants at birth. Fifty-two of the successfully vaccinated babies were revaccinated at from 13 to 16 months of age, and 30.8 per cent of them again exhibited primary takes. The facts just mentioned are not generally appreciated and indicate that in many countries revaccinations are not practiced with sufficient frequency and regularity.

It may be found that the immunity induced by one intradermal inoculation of culture virus will not be as enduring as that produced by potent calf lymph. If such proves to be the case, the advantages of the intradermal method of administration are so obvious and the amount of reaction and inconvenience caused by the culture virus is so inconsequential that two or more intradermal inoculations, made at the same time in different areas of skin, or more frequent revaccinations may meet with general favor as means of prolonging or renewing an immunity to smallpox.

This discussion should not be concluded without calling attention to the fact that the direct evidence in our possession only shows that the culture virus will prevent an infection with calf lymph. Such evidence leads us to believe that individuals vaccinated with culture virus are protected against smallpox. We cannot be absolutely certain that this is true, however, until people vaccinated with the culture virus have been exposed to smallpox.

#### CONCLUSIONS

The second revived strain of culture vaccine virus has been propagated through 130 culture passages during a period of 3 years. It seems to be adapted to *in vitro* cultivation and still has an intradermal titer (rabbits) of 1 to 100,000 or 1 to 1,000,000.

Intradermal inoculations in human beings of 0.1 cc. amounts of culture virus diluted from 2.5 to 10 times result in primary takes in unvaccinated people and immune reactions or accelerated takes in individuals previously successfully vaccinated. Primary takes produce an immunity to standard strains of calf lymph.

Culture virus mixed with purified gum acacia (2.5 per cent), frozen, desiccated, and sealed *in vacuo* retains its activity for a month at 37°C., and when the dried virus is resuspended in saline solution it is suitable for intradermal vaccination of human beings.

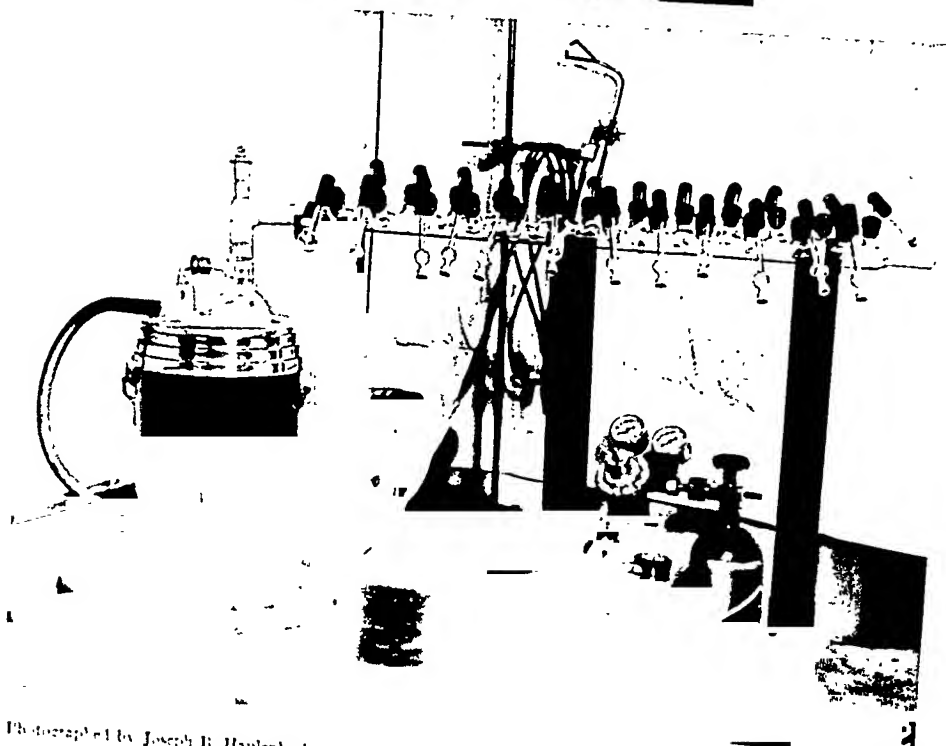
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#### EXPLANATION OF PLATE 25

FIG. 1. Reaction produced in the skin of a young child by culture vaccine virus introduced intradermally. The lesion made its appearance 10 days after inoculation and was photographed 2 days later.

FIG. 2. The Mudd-Flosdorf drying apparatus used in the preservation of culture vaccine virus to be employed for intradermal vaccination of human beings. The tubes containing the virus are closed at the distal end with rubber stoppers. Later we decided that glass containers constructed so that rubber stoppers would not be needed are preferable.



Photographed by Joseph R. Haulenbeck

(Experiments on the



# THE INFECTION OF MICE WITH SWINE INFLUENZA VIRUS

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Andrewes, Laidlaw and Smith (1), and Francis (2), have succeeded in infecting white mice with a virus isolated from human cases of influenza. The descriptions of the disease produced in the two laboratories indicate a complete agreement in results. The former workers further observed that the virus of swine influenza also was transmissible to mice and produced in these animals a disease similar in all respects to that caused by the virus of human origin.

Because of the obvious advantages of the use of mice over either ferrets or swine for certain phases of work with swine influenza, it has seemed advisable to study the mouse disease thoroughly. It appeared important to know whether mice could be infected directly from swine, as ferrets can (3 and 4), whether the virus is modified for swine by mouse passage and whether observations concerning the mouse disease might be directly applicable to the swine disease. Furthermore, study of swine influenza infection of mice offered certain advantages over similar studies with the virus of human origin because with the swine virus it is possible to revert, when occasion requires, to the natural host. The present paper confirms and extends the observations of Andrewes, Laidlaw and Smith (1) regarding the infectivity of swine influenza virus for white mice.

## EXPERIMENTAL

*Method Used in Inoculating Mice Intranasally.*—Through a personal communication from Drs. Andrewes, Laidlaw and Smith, prior to a description of their technic, it was learned that white mice could be infected with swine influenza virus. Consequently the method of inoculation developed in this laboratory differs from theirs.

Mice to be infected were etherized in a glass jar until they fell on their sides.



Their noses and mouths were then immersed in the virus suspension, contained in one side of a slightly tilted Petri dish. They were kept thus submerged for 3 or 4 seconds and during this time inspired from 7 to 10 times. It was important to ascertain that the animals' respirations continued during the entire time their noses were in the virus suspension. By measuring the volume of suspension before and again at the end of experiments in which large series of mice were infected from the same container, it was found that each mouse removed an average of 0.14 cc. of suspension. Since much of the suspension adhered to the fur about the nose and mouth, the average dose of virus gaining entrance to the respiratory tract by this method of infection probably lay somewhere between 0.05 cc. and 0.1 cc. This dosage is slightly greater than that used by Andrewes, Laidlaw and Smith in most of their experiments. The method of inoculation just described has been carefully controlled and found to be harmless in itself.

Attempts to produce recognizable disease in unanesthetized mice have been uniformly unsuccessful owing obviously to the fact that the unanesthetized animals hold their breath during the time that their noses are submerged in the virus suspension. While mice treated in this way develop no clinical evidence of illness and show no pulmonary lesions when autopsied 4 to 6 days later, they may become immunized and resist later infection with virus administered in the usual fashion.

In the experiments to be described, the virus suspensions employed in inducing infections were, unless otherwise specified, the supernatant fluid from sedimented but uncentrifuged 5 per cent suspensions of lungs from infected animals. In infecting mice or ferrets, swine influenza virus alone was employed; while in infecting swine the virus was mixed with a small amount of a culture of *H. influenzae suis* (5) in order to produce typical swine influenza (6). Animals which died or were killed on the 3rd or 4th day following inoculation were found to furnish the most satisfactory virus.

#### *The Production of Disease in White Mice by Intranasal Inoculation with Infectious Material from Cases of Swine Influenza*

Two field strains of the virus have been tested for their ability to produce disease in mice. Since they differed somewhat in initial pathogenicity for mice, they will be discussed separately. Strain 15, obtained from Iowa in December, 1930, and maintained for study in this laboratory by serial transfer through swine at least once every 90 days, proved regularly pathogenic for mice. There was nothing to indicate that a preliminary adaptation period was essential to the acquisition of full virulence of this strain for mice. Passage directly from swine to mice induced a disease clinically identical with that already described by Andrewes, Laidlaw and Smith (1).

The incubation period ranged from 24 to 48 hours. The first symptoms were loss of appetite and malaise. Infected mice huddled in a corner of their cages and their coats were roughened. By the following day exaggerated respiratory movements were apparent and sounds similar to fine crepitant râles could be heard by listening over the cage. Deaths occurred as early as the 3rd day and usually by the 8th day all mice had succumbed. Animals sacrificed on the 3rd or 4th day exhibited plum-colored areas of pulmonary consolidation involving from 1/4th to 3/4ths of the total lung volume. Animals allowed to proceed to death exhibited, as a rule, a complete pneumonia indicating that the lung lesions were progressive in character. The pathological picture was identical with that already described (1 and 2). The mortality rate among mice infected with fresh unglycerolated Strain 15 virus obtained directly from swine approaches 100 per cent.

Strain 20 virus was obtained from Iowa in December of 1934 and, so far, has behaved differently from Strain 15 in its initial pathogenicity for mice. This strain has undergone only three serial transfers in swine since being brought to the laboratory. Mice inoculated with Strain 20 virus directly from swine show little evidence of illness. Their fur may become a bit rough on the 3rd to 6th day after infection, but they do not become seriously ill and none die. If they are sacrificed on the 4th day, the plum-colored areas of pulmonary consolidation, characteristic of influenza virus infection in mice, are seldom seen. The lungs either appear normal or are very slightly hyperemic and exhibit one or two small areas of consolidation. However, serial passage from these mice soon yields a virus that is as regularly lethal for mice as Strain 15. Strains 15 and 20 are immunologically identical as judged by cross-protection and cross-neutralization tests. Also both produce a characteristic pneumonia in ferrets (4). The difference in initial pathogenicity for mice may be in some way referable to the prolonged and frequent serial transfer of Virus 15 through swine. It will therefore be of interest to observe whether Virus 20, after more serial transfers through swine, acquires the ability to cause fatal initial infections in mice.

A record of some experiments in which Strain 15 and Strain 20 virus were transferred serially in mice is given in Table I. The virus used in initiating these experiments was either from swine or ferrets. The swine virus had undergone no ferret or mouse passages since coming to the laboratory. The ferret virus had been derived originally from swine but had been submitted to no mouse passages. Virus for infections beyond the first mouse passage was derived from the lungs of mice dead or killed on the 4th day postinfection. Mice sacrificed for virus were, of course, not included in the record of the experiments outlined in Table I.

As shown by the table, Strain 15 virus produced fatal infections in

mice from the 1st passage. Strain 20 virus, on the other hand, required at least 2 mouse passages to bring it to full pathogenicity for mice. It was at first thought that this might be due merely to a difference in the virus content of infected swine and mouse lungs. To eliminate this possibility Strain 20 virus was transferred serially 4 times through mice until it was fully pathogenic for this species. It was then passed through a pig and virus recovered from the swine lung was used to infect mice. All of 6 mice inoculated with this

TABLE I

*The Serial Passage of Swine Influenza Virus in White Mice*

| Source of virus                | Strain of virus | Serial passages in white mice |           |          |                     |           |          |                     |           |          |                     |           |          |
|--------------------------------|-----------------|-------------------------------|-----------|----------|---------------------|-----------|----------|---------------------|-----------|----------|---------------------|-----------|----------|
|                                |                 | 1st                           |           |          | 2nd                 |           |          | 3rd                 |           |          | 4th                 |           |          |
|                                |                 | No. inocu-<br>lated           | No. dying | Survival | No. inocu-<br>lated | No. dying | Survival | No. inocu-<br>lated | No. dying | Survival | No. inocu-<br>lated | No. dying | Survival |
| Lung                           |                 |                               |           |          |                     |           |          |                     |           |          |                     |           |          |
|                                |                 |                               |           | days     |                     |           | days     |                     |           | days     |                     |           | days     |
| Swine 1574 (fresh).....        | 15              | 5                             | 5         | 5-7      | 5                   | 5         | 4-6      | 9                   | 9         | 3-8      |                     |           |          |
| Swine 1574 (glycerolated)..... | 15              | 6                             | 4         | 6-8      | 9                   | 9         | 4-7      | 5                   | 5         | 5-8      | 7                   | 7         | 4-6      |
| Ferret 66 (fresh).....         | 15              | 3                             | 3         | 3-4      |                     |           |          |                     |           |          |                     |           |          |
| Swine 1610 (fresh).....        | 15              | 4                             | 4         | 4-8      |                     |           |          |                     |           |          |                     |           |          |
| Swine 1601 (fresh).....        | 15              | 5                             | 5         | 3-6      |                     |           |          |                     |           |          |                     |           |          |
| Swine 1616 (fresh).....        | 15              | 5                             | 3         | 7-12     |                     |           |          |                     |           |          |                     |           |          |
| Swine 1550 (glycerolated)..... | 20              | 6                             | 0         |          | 5                   | 0         |          | 5                   | 3         | 6-13     | 7                   | 7         | 4-9      |
| Swine 1575 (fresh).....        | 20              | 6                             | 0         |          | 5                   | 4         | 5-8      | 9                   | 9         | 4-7      | 5                   | 5         | 6-8      |
| Ferret 86 (fresh).....         | 20              | 5                             | 0         |          | 8                   | 7         | 4-6      | 6                   | 5         | 3-7      |                     |           |          |
| Swine 1624 (fresh).....        | 20              | 8                             | 0         |          | 7                   | 1         | 6        | 6                   | 6         | 4-7      |                     |           |          |

swine passage virus succumbed typically in between 4 and 7 days. This experiment indicated that an actual increase in virulence of Strain 20 for mice had occurred and that the change was not reversed by one back-passage through swine.

#### *Bacteriology and Filtration Experiments*

Bacteriological study of the pneumonic lungs of mice dead following infection with the virus has not suggested that any single bacterial component played a rôle in the mouse disease. Usually the lungs have been sterile. When bacteria were encountered there was nothing to

indicate that they had enhanced the severity of the disease and seldom was the same organism recovered from animals of two succeeding serial transfers. No single bacterial form has been found with any degree of constancy. It was of interest to note that, while *H. influenzae suis*, essential to the production of influenza in swine (6), was present in the swine material used in initiating many of the mouse infections it failed to become established in mice.

Bacteriologically sterile Berkefeld N filtrates of suspensions of the lungs of either infected swine or mice have been found fully capable of infecting mice when administered in the customary fashion. Usually mice infected with filtrates have succumbed. However, in some instances the illness in the filtrate-infected animals has been less severe than in the controls receiving unfiltered suspension. This was undoubtedly due to the loss of some virus by adsorption during filtration, because mice of the succeeding passage have regularly succumbed. From these experiments it is apparent that in mice the swine influenza virus is capable of inducing an extensive and fatal pneumonia unaided by secondary bacterial invaders. The mouse disease thus differs materially from that seen in swine in which not only the virus but a bacterium, *H. influenzae suis*, are etiologically essential (6).

#### *Immunity Conferred by Infection*

Mice surviving infection with swine influenza virus cannot be reinfected for a period of at least a month. It is not known how much longer their immunity may endure. The production of an extensive pneumonia is not essential to immunization. Mice that have been infected with Strain 20 virus directly from swine and that develop no pneumonia are apparently as solidly immune to Strain 15 or 20 virus as are animals that survive only after a prolonged and stormy pneumonia convalescence.

#### *Failure of Contact Transfer in Mice*

Normal mice placed in the same cages with those infected with swine influenza virus have in no instance become recognizably ill. Furthermore, mice exposed in this way have proven fully susceptible to infection when later inoculated intranasally with virus under ether anesthesia.

In one experiment bread soaked in swine influenza virus of full pathogenicity was fed to a group of 10 mice four times during the course of 6 days. The animals ate of the mixture and the shavings used as bedding became moist with it. None of the mice became ill, and 14 days after the last virus feeding all were tested for immunity by intranasal inoculation with virus. All proved fully susceptible and died as promptly as their controls. These experiments confirm the observation of Andrewes, Laidlaw and Smith (1) that swine influenza virus does not produce a readily communicable disease in mice.

*Failure of the Virus to Infect Mice When Administered Subcutaneously or Intraperitoneally*

Mice, inoculated subcutaneously or intraperitoneally with 0.2 cc. amounts of virus known to be fatally pathogenic by nose, exhibited no clinical evidence of illness and were completely negative when autopsied 4 to 6 days following inoculation. This is in accord with the experience of Andrewes, Laidlaw and Smith (1) and indicates a tropism of the virus for respiratory tract tissues similar to that seen in swine (7). Immunity following repeated subcutaneous or intraperitoneal administrations of virus will be discussed in a later paper.

*The Infection of Swine and Ferrets with Mouse-Passaged Virus*

It has previously been reported (4) that 16 serial transfers of the swine influenza virus in ferrets failed to alter its pathogenicity for swine. Since that time 4 more serial ferret passages have been tested. The hog receiving 19th passage virus mixed with *H. influenzae suis* failed to become typically ill. However, swine inoculated intranasally with 20th, 23rd and 24th ferret passage virus together with *H. influenzae suis* developed characteristic swine influenza. From this it would appear that 24 serial transfers of the virus in ferrets had not altered its pathogenicity for swine.

Similarly, prolonged serial passage of swine influenza virus in mice has exerted no appreciable influence on its virulence or infectivity for swine. Fourth, 8th, 16th, 23rd, 36th, 41st, 45th and 53rd mouse passage Strain 15 virus mixed with *H. influenzae suis* has been administered intranasally to swine. All 8 pigs inoculated developed characteristic swine influenza indistinguishable in any respect from that induced by similar inoculation with virus of swine origin. No significant lengthening of the incubation period, decrease in clinical

severity or diminution in the extent of the pathological alterations encountered at autopsy was observed in these swine. The virus was also found to be fully pathogenic for ferrets after 16 and 46 mouse passages. Virus 20, after 4 mouse passages, proved fully pathogenic for a hog when administered intranasally with *H. influenzae suis*. These experiments indicate that prolonged serial passage of swine influenza virus in mice does not attenuate it for swine. They moreover seem to prove that the agent responsible for the disease in mice is actually the swine influenza virus.

Further evidence as to the identity of the virus causing disease in mice with the swine influenza virus was furnished by cross-neutralization experiments. In these, virus of swine origin that had at no time been submitted to mouse passage, and convalescent serum from swine infected with such virus were used. It was found that such convalescent swine sera neutralized mouse passage virus in either mice or swine as well as swine virus in mice. Furthermore, sera from recovered mice neutralized swine virus. It would thus seem established that the agent causing the disease in mice is the swine influenza virus and not some intercurrent infectious agent acquired during serial mouse passage.

#### *Titration of Swine Influenza Virus in Mice*

In certain types of experiments exact knowledge as to the minimal infectious dose of a virus is desirable. It was hoped that, with the mouse available as a test animal, accurate quantitative experiments with swine influenza virus might be possible. With this end in view mouse passage, ferret passage and swine passage virus were titrated in mice. From a group of four such experiments conducted in November of 1934, using 3 mice per dilution, it was found that mouse passage virus was active in a final dilution of 1:20,000 (on the basis of wet lung weight), and that ferret and swine passage virus were only slightly if at all less active. There was no further occasion to titrate the virus until February of 1935. At this time the final infecting dilution was found to be 1:2000. In April and May titrations, conducted in the same manner as above, indicated that the final infecting dilution for both mouse and swine passage virus was 1:200. The mice in all the titration experiments were from the same stock and all were used

within a week of the time weaned, being thus of approximately the same age. There is no explanation apparent for the wide variation found in the minimal infectious dose of the virus. It is suggested that it may be a seasonal variation but only titrations throughout several years can establish this. From this standpoint it is of interest that November, the month in which swine influenza virus appeared to be most highly infectious for mice, is also the month in which the middle western swine epizootics most frequently appear. The experiments indicate, aside from this possible epidemiological interest, that titrations of virus, to be valid for a given experiment, must be done at the same time as the experiment for which the data are intended.

#### *Immunological Relationship of Swine and Human Influenza Virus*

It has been noted by Smith, Andrewes and Laidlaw (3 and 8) that the infection of ferrets with either swine or human influenza virus confers considerable reciprocal protection against the other virus. Cross-neutralization tests, however, have shown that, while the two viruses are related, they are not identical. Francis (9) found that the sera of neither ferrets nor swine recovered from swine influenza were capable of neutralizing his PR 8 strain of the human virus although they were known to be effective against the swine virus. In like manner, Laidlaw, Smith, Andrewes and Dunkin (10) have noted that the serum of a horse, hyperimmunized against the swine influenza virus, was highly active against its homologous virus but did not neutralize their human strains in the dilutions tested. Neutralization in the opposite direction was, however, somewhat better, for the serum of a horse hyperimmunized against the human virus neutralized swine virus in the lower dilutions.

Experiments conducted in this laboratory are in agreement with the work just cited. Mice immunized by Francis against his PR 8 and Philadelphia strains of the human virus were found resistant to a dose of swine influenza virus that killed all controls in the experiment. In like manner, mice immunized against swine influenza virus in this laboratory were found resistant when tested with a dose of PR 8 human virus that proved fatal for 5 out of 6 controls. Serum from a horse hyperimmunized by Laidlaw, Smith, Andrewes and Dunkin (10) against their W.S. strain human virus and from a rabbit hyper-

immunized by Francis against his PR 8 human virus proved capable of completely neutralizing swine influenza virus for mice. The control mice in these experiments received swine influenza virus mixed with normal horse or normal rabbit serum and all died on the 3rd and 4th day following inoculation.

#### DISCUSSION

The disease produced in mice by infection with swine influenza virus resembles that in ferrets (3 and 4). It differs materially from that induced or occurring naturally in swine. In mice and ferrets the virus administered intranasally suffices to produce an extensive and often fatal pneumonia. In swine, however, a severe illness, characteristic of the naturally occurring influenza in this species, ensues only when the virus is administered in company with a bacterium, *H. influenzae suis* (6). The mouse and the ferret must therefore be considered as highly artificial hosts in that in neither species is the disease etiologically a complete replica of swine influenza; there is no evidence that *H. influenzae suis* or any other organism contributes significantly to their illness.

The virus infection in mice appears to be non-contagious, while the ferret disease is communicable (3), and influenza in swine is highly contagious. This difference is a useful one from an experimental standpoint for with mice the time and space consuming practise essential to isolation is unnecessary.

Evidence of adaptation of swine influenza virus to mice was noted with one of the two strains studied. Strain 20 virus, while initially infectious for mice, required several serial mouse passages to bring it to full pathogenicity for this species. Strain 15 virus, however, required no adaptation to mice. It killed quite regularly even in its first serial mouse passage, and repeated transfers in mice have not noticeably enhanced its activity for this species. There was no evidence that prolonged serial passage of swine influenza virus in mice attenuated it for its natural host.

It seems clear from the present experiments that the swine influenza virus is a stable one, so far as its three known hosts are concerned; for prolonged passage through ferrets has not altered its pathogenicity for mice or swine, and its infectivity and virulence for



ferrets or swine are unaffected by repeated serial transfers in mice. It can be transferred at will from any one of its known animal hosts to any other, and no significant alteration in its properties, other than an enhancement in the virulence of Strain 20 for mice by serial passage in this species, has been noted. The swine influenza virus would thus appear to differ significantly from strains of the human influenza virus so far studied, both as concerns its initial pathogenicity for ferrets and its infectivity for mice. The experience with human influenza virus has indicated that it undergoes an adaptation during early passages in ferrets. Francis (2) noted that in etherized ferrets his PR 5 human virus strain did not produce pneumonia until its 6th serial passage. He remarked that the disease then developing more closely resembled that produced in etherized ferrets by swine influenza virus (4) than the classical disease first described by Smith, Andrewes and Laidlaw (3). The latter workers have since found that serial passage of their human virus in anesthetized ferrets also eventually results in the appearance of pneumonia in animals inoculated in this way (11). It would thus appear that only after a number of serial passages in ferrets does the human influenza virus acquire the ability, possessed by the swine influenza virus from the very outset, of producing pneumonia in ferrets.

Another initial difference in the two viruses that disappears after the human strain has been transferred several times, has to do with its infectivity for mice. Human influenza virus is said not to be infectious for mice until after transfer serially through ferrets (2 and 11), while the swine influenza virus requires no intervening ferret passages to become established in mice. This difference is an interesting one for it suggests that passage of human influenza virus through ferrets alters it in such a way that it becomes more like the swine influenza virus and less like the virus originally obtained from the human patient. The acquisition by human influenza virus, upon ferret passage, of pathogenic properties for ferrets and mice similar to those possessed from the outset by swine influenza virus, suggests that the human virus undergoes changes as a result of passage in animals that the swine influenza virus has perhaps already undergone.

Laidlaw (11) has recently suggested that the swine influenza virus may represent the virus of the human pandemic of 1918 which at that

time in some way became established in swine and has since persisted as the cause of an epizootic disease in this species. If this should be the case, the initial differences, aside from the immunologic ones, between it and recently isolated human strains may be those due to "fixation" by prolonged sojourn in a foreign host.

#### SUMMARY

The experiments confirm the earlier observation of Andrewes, Laidlaw and Smith that the swine influenza virus is pathogenic for white mice when administered intranasally. Two field strains of the swine influenza virus were found to differ in their initial pathogenicity for mice. One strain was apparently fully pathogenic even in its 1st mouse passage while the other required 2 or 3 mouse passages to acquire full virulence for this species. Both strains, however, were initially infectious for mice, without the necessity of intervening ferret passages. There is no evidence that bacteria play any significant rôle in the mouse disease though essential in that of swine, and fatal pneumonias can be produced in mice by pure virus infections. Mice surviving the virus disease are immune to reinfection for at least a month. In mice the disease is not contagious though it is notably so in swine. The virus, while regularly producing fatal pneumonias when administered intranasally to mice, appears to be completely innocuous when given subcutaneously or intraperitoneally. Prolonged serial passage of the virus in mice does not influence its infectivity or virulence for swine or ferrets. It is a stable virus so far as its infectivity is concerned, and can be transferred at will from any one of its three known susceptible hosts to any other.

In discussing these facts the stability of the swine influenza virus has been contrasted with the apparent instability of freshly isolated strains of the human influenza virus. Though the mouse is an unnatural host for the virus it is, nevertheless, useful for the study of those aspects of swine influenza which have to do with the virus only.

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# CUTANEOUS REACTIVITY OF IMMUNE AND HYPERSENSITIVE RABBITS TO INTRADERMAL INJECTIONS OF HOMOLOGOUS INDIFFERENT STREPTOCOCCUS AND ITS FRACTIONS

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It is now well established that rabbits can be made hypersensitive by repeated intracutaneous injections of streptococci so that subsequent inoculations induce enormously exaggerated skin lesions (1-6); and it is also known that rabbits immunized by suitable intravenous injections of the same microorganisms show lesions smaller than normal at subsequent skin tests (7, 8). The purposes of the present study were: (a) to observe the types of dermal reactions induced in such bacterially hypersensitive and immune rabbits by different chemical fractions of the homologous cocci, and (b) to investigate the relationship between these reactions and the circulating antibodies. The work was undertaken in three separate experiments, but, since the basic technique was the same in each, the methods and results will be described together, with special notation where exceptions were encountered.

## EXPERIMENTAL

The bacterial culture used throughout was a strain (Q 155/0/7) of Type I indifferent streptococcus (9) which had been extensively tested in this laboratory.

Healthy adult Chinchilla rabbits were used; all in Experiment 1 were females, while all in Experiments 2 and 3 were males. In each experiment the animals were divided into five groups which were treated as follows: Group A comprised normal controls; Group B was sensitized by repeated intracutaneous inoculations of intact cocci; the remaining three groups were immunized by intravenous injections—Group C received intact cocci, Group D, the so called nucleoprotein fraction, and Group E an emulsion of mechanically ground bacterial bodies. The number of rabbits in each of the three experiments is summarized in Table I.

After test bleedings or skin tests had indicated that the animals were sufficiently immunized or sensitized, sera were collected for final titrations of agglutinins and

of precipitins against both type- and non-type-specific streptococcal fractions. Following depilation, each rabbit was skin-tested with intracutaneous injections of (a) 0.01 and 0.001 cc. of a living 20 hour broth culture of *Streptococcus* Q 155/0/7; (b) 0.5 and 0.1 mg. of Q 155 "nucleoprotein"; and (c) 2.5 and 0.25 mg. of Q 155 type-specific substance. In each instance the dilution was such that the injected material was contained in a volume of 0.1 cc. In Experiments 1 and 2 all test substances were injected simultaneously, but in Experiment 3 the tests with nucleoprotein were made 48 hours after the others. The volumes of all resultant lesions were calculated daily over a period of 2 weeks and were charted for comparison.

*Preparation of Nucleoprotein Fraction.*—The non-type-specific nucleoprotein fraction, here referred to as P, after Lancefield's terminology, was prepared from *Streptococcus* Q 155/0/7 by a slight modification of the method described by

TABLE I  
*Rabbits in Each Group in the Three Experiments*

| Group   | Number of rabbits in |        |        | Total |
|---|----------------------|--------|--------|-------|
|   | Exp. 1               | Exp. 2 | Exp. 3 |       |
| A Normal controls   |                      |        |        |       |
| B Sensitized intracutaneously with intact cocci           | 3                    | 4      | 6      | 13    |
| C Immunized intravenously with intact cocci               | 3                    | 4      | 5      | 12    |
| D Immunized intravenously with nucleoprotein (P)          | 3                    | 5      | 7      | 15    |
| E Immunized intravenously with bacterial emulsion (B. E.) | 0                    | 4      | 4      | 8     |
|   | 3                    | 3      | 4      | 10    |

Lancefield (10). It was not obtained in dry form, however, but was standardized, instead, by micro Kjeldahl determinations. Furthermore, on the advice of Dr. Lancefield, N/1000 sodium hydroxide rather than N/100 was used for the extraction of the ground cocci. The yield was approximately 28 mg. of P from each liter of culture.

*Preparation of the Type-Specific Carbohydrate.*—The source of this material, referred to as S, was the clear supernatant left over in the preparation of P when NaOH extracts were acidified and the resultant precipitate centrifuged off. It was further alkalized and reprecipitated several times to remove additional protein and the final supernatant was made neutral to phenol red. The clear yellow fluid thus obtained was concentrated to small volume by vacuum distillation at 25°C. This was then treated with three volumes of 95 per cent alcohol in the cold for 3 hours, and the white precipitate was redissolved in saline. As the supernatant revealed no S when tested with anti-S serum, it was discarded. Alcohol precipitation was repeated four times and the final precipitate was dried over P<sub>2</sub>O<sub>5</sub> and weighed. In Experiment 3 an additional procedure was employed:

the supernatant after acid precipitation was passed through an ultrafilter. The filtrate thus obtained was concentrated by vacuum distillation at 25°C. and then submitted to alcohol precipitation as noted above. As shown by precipitin tests with anti-P serum (Table V), the S extract used in Experiment 2 contained minute traces of P, but that employed in Experiment 3 probably did not.

*Preparation of Bacterial Emulsion (B. E.).*—20 hour living cultures of *Streptococcus* Q 155/0/7 in 0.05 per cent dextrose broth were centrifuged and the frozen and dried sediment was ground in a ball-mill until only Gram-negative debris could be seen in stained films, and culture gave no growth. The powder was weighed and added to physiologic saline in standard amounts. Some went into solution, but much was in suspension so that it was necessary to mix thoroughly before it was used for intravenous injection.

*Method of Sensitization.*—The rabbits were sensitized by intracutaneous injections of 0.001 cc. living culture of *Streptococcus* Q 155/0/7 diluted in Ringer's

TABLE II  
*Amount of Antigen and Route of Injection in Each Group*

| Group                      | Material injected          | Route       | Total amount received by each animal |           |           |
|----------------------------|----------------------------|-------------|--------------------------------------|-----------|-----------|
|                            |                            |             | Exp. 1                               | Exp. 2    | Exp. 3    |
| B                          | Intact cocci               | Intradermal | 0.121 cc.                            | 0.017 cc. | 0.017 cc. |
| C                          | " "                        | Intravenous | 215.0 cc.                            | 169.0 cc. | 139.0 cc. |
| D                          | Nucleoprotein (P)          | "           | Not done                             | 944.0 mg. | 442.0 mg. |
| E                          | Bacterial emulsion (B. E.) | "           | 421.0 mg.                            | 380.0 mg. | 564.0 mg. |
| Days over which given..... |                            |             | 255                                  | 90        | 105       |

solution so that the desired amount was contained in 0.1 cc. These inoculations were given daily until the animals showed hypersensitive reactions to them, which usually occurred in 10 to 15 days. Following this, sensitivity was maintained by single weekly injections.

*Method of Immunization.*—In Experiments 1 and 2 intravenous injections were given on 4 consecutive days each week, with no injections the remaining 3 days. In Experiment 3, injections were given daily every alternate week. The animals receiving the intact cocci were started on small doses of heat-killed vaccine and were continued on the centrifuged sediment of living 20 hour broth cultures resuspended in saline. Those immunized with P were started on doses of 10 mg. daily which was increased to 20 mg. The rabbits immunized with B. E. received 2.5 mg. daily at first which was increased to 25.0 mg. daily. The total amounts received by the individual animals of the three immune groups, together with the total time immunization was continued, are shown in Table II.

*Technique of Agglutinin Tests.*—0.5 cc. of each serum dilution was mixed with

0.5 cc. of a 20 hour broth culture of *Streptococcus* Q 155/0/7, and readings were made after 1 hour at 56°C. and 20 hours in the ice box.

*Precipitin Tests.*—0.5 cc. of each dilution of P and S respectively was mixed with 0.1 cc. of serum and readings were made after 15 minutes for ring reactions and after 2 hours at 37°C. and 20 hours in the ice box.

### *Results of Serologic Study*

*Agglutinins.*—The agglutination results are summarized in Table III. As expected, the rabbits immunized with intact cocci showed

TABLE III  
*Summary of Results of Agglutinin Tests*

| Group   | No. of rabbits | Agglutinin titer |      |      |      |      |       |       |       |        |        |
|---|----------------|------------------|------|------|------|------|-------|-------|-------|--------|--------|
|   |                | Negative         | 1-10 | 1-20 | 1-40 | 1-80 | 1-160 | 1-320 | 1-640 | 1-1280 | 1-2560 |
| A Normal controls   | 11             | 8                | 3    |      |      |      |       |       |       |        |        |
| B Sensitized intracutaneously with intact cocci           | 9              |                  |      |      |      | 1    | 4     | 4     |       |        |        |
| C Immunized intravenously with intact cocci               | 15             |                  |      |      |      |      |       |       |       | 1      | 10     |
| D Immunized intravenously with nucleoprotein (P)          | 8              |                  |      |      |      |      | 2     | 6     |       |        |        |
| E Immunized intravenously with bacterial emulsion (B. E.) | 10             | 2*               |      | 1*   |      |      | 3     | 0     | 4     |        |        |

Figures indicate the number of rabbits in the respective groups showing agglutination to the titer designated.

\* These rabbits all in Experiment 1.

high titers. Those intracutaneously sensitized, and the P-immune and B.E.-immune groups all showed comparatively low titers which were, however, distinctly greater than normal except in the case of the three B.E.-immune rabbits of Experiment 1. These differed from the seven corresponding animals of Experiments 2 and 3 in that their sera gave negative, or essentially negative, agglutination reactions. The low agglutinin titers in these three animals is difficult to explain since each received a total of 421 mg. of B.E. compared with 380 mg. given in Experiment 2; possibly the reason lies in the fact that in Experiment 1 immunization was performed with smaller individual doses over a much longer period.

*Anti-P Precipitins.*—As seen in Table IV, the sera of normal rabbits and of those sensitized with small intracutaneous inocula of living culture gave no precipitation with P extract in any of the dilutions used. As has been noted by others (11), the animals immunized with large intravenous doses of intact cocci varied greatly in titer; eight gave negative results, while others showed relatively high titers. As expected, the P-immunized animals gave uniformly high titers; less expected was the finding that the B.E.-immunized group gave results

TABLE IV  
*Summary of Results of Precipitin Tests Using P as Antigen*

| Group   | No. of rabbits | Precipitin titer |       |       |       |        |        |        |          |          |          |
|---|----------------|------------------|-------|-------|-------|--------|--------|--------|----------|----------|----------|
|   |                | Negative         | 1-240 | 1-480 | 1-960 | 1-1920 | 1-3840 | 1-7680 | 1-15,360 | 1-30,720 | 1-61,440 |
| A Normal controls   | 6              | 6                |       |       |       |        |        |        |          |          |          |
| B Sensitized intracutaneously with intact cocci           | 9              | 9                |       |       |       |        |        |        |          |          |          |
| C Immunized intravenously with intact cocci               | 15             | 8                |       | 1     |       | 2      | 1      | 2      | 1        |          |          |
| D Immunized intravenously with nucleoprotein (P)          | 8              |                  |       |       |       |        |        |        | 3        | 1        | 4        |
| E Immunized intravenously with bacterial emulsion (B. E.) | 10             | 1                |       |       |       | 1      |        | 1      | 4        | 2        | 1        |

Figures indicate the number of rabbits in the respective groups showing precipitation to the titer designated.

of almost the same degree. All precipitates were of the flocculent type encountered with bacterial proteins.

*Anti-S Precipitins.*—The results of these tests are summarized in Table V. Here again, the normal and the intracutaneously sensitized animals were negative. In contrast, the animals immunized by means of intravenous injections of intact cocci showed high anti-S titers, the precipitates formed being of the disc type characteristic of carbohydrate antigens.

Four of the eight P-immunized rabbits showed negative reactions, and in the remainder the precipitates merely formed slight whorls on agitation; these reactions were probably due to traces of P in the S



extract. In this regard it is important, for reasons to be mentioned later, to point out that in Experiment 3 positive precipitation with S extract occurred with the serum of only one rabbit immunized with P, and this reaction was questionable (see footnote to Table V). The results in the B.E.-immunized group were negative in most instances; the serum of only one animal gave a reaction which in titer or character suggested the presence of anti-S precipitins.

TABLE V  
*Summary of Results of Precipitin Tests Using S as Antigen*

| Group   | No. of rabbits | Precipitin titer |        |        |        |        |          |          |          |
|---|----------------|------------------|--------|--------|--------|--------|----------|----------|----------|
|   |                | Negative         | 1-1200 | 1-2400 | 1-4800 | 1-9600 | 1-19,200 | 1-38,400 | 1-76,800 |
| A Normal controls   | 7              | 7                |        |        |        |        |          |          |          |
| B Sensitized intracutaneously with intact cocci           | 9              | 9                |        |        |        |        |          |          |          |
| C Immunized intravenously with intact cocci               | 12             |                  |        |        |        |        | 2        | 2        | 7        |
| D Immunized intravenously with nucleoprotein (P)          | 8              | 4                | 3*     | 1      |        |        |          |          |          |
| E Immunized intravenously with bacterial emulsion (B. E.) | 7              | 5                |        | 1      |        | 1      |          |          |          |

Figures indicate the number of rabbits in the respective groups showing precipitation to the titer designated.

\* Includes the only animal of Experiment 3 giving a positive reaction and this was merely  $\pm$ .

### *Results of Skin Tests*

The results of the skin tests of Experiment 3 are recorded graphically in Chart 1, in which the curves represent the average volumes of the lesions in each group of animals from day to day. The results were of the same general character in all three experiments, but in Experiments 1 and 2, because of the simultaneous intradermal injection of all the test substances,<sup>1</sup> the lesions of the intracutaneously sensitized animals were less striking than in Experiment 3.

<sup>1</sup> It has been found in work still in progress that injection of P, even intradermally, results in the desensitization of intracutaneously sensitized rabbits.

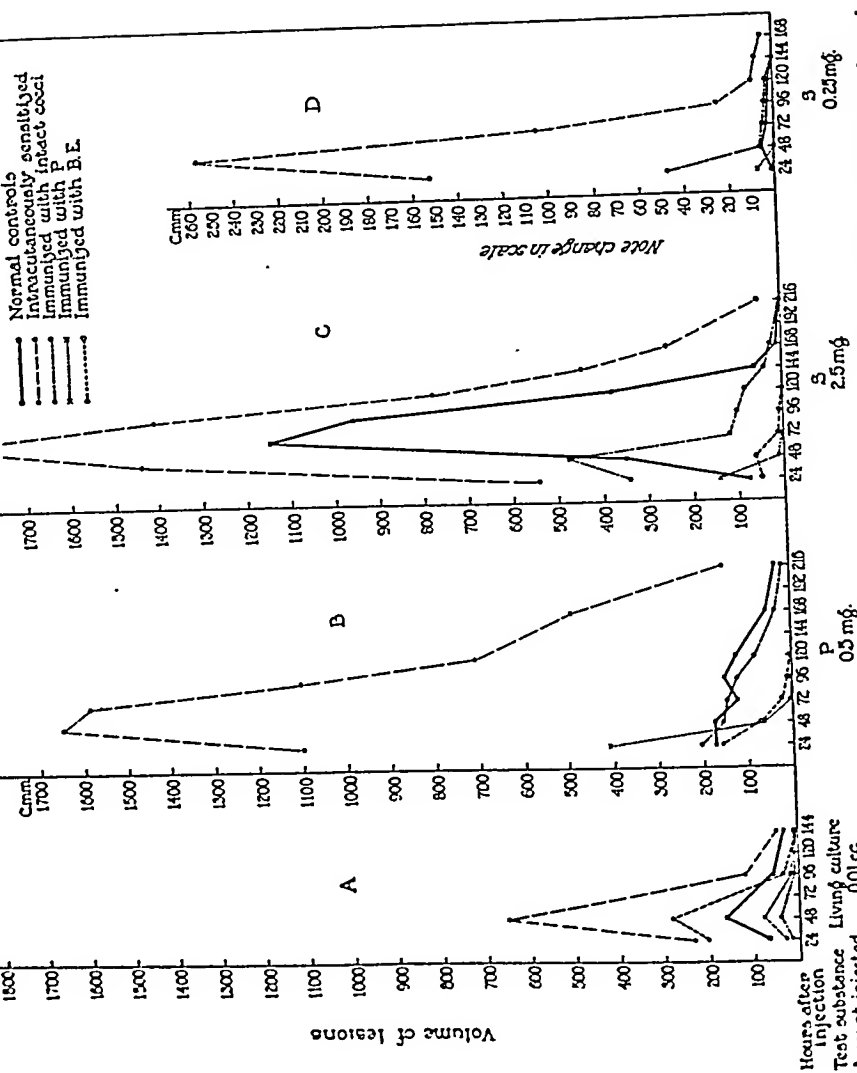


CHART 1. Volume curves of dermal reactions induced by Streptococcus Q 155 and its fractions in rabbits of the different groups of Experiment 3.

*Intact Cocci.*—In Chart 1A are shown the averages of the cutaneous lesions induced in the animals of Experiment 3 by the intradermal injection of 0.01 cc. of living 20 hour broth culture of Streptococcus Q 155/0/7. The exaggerated skin reactions of the intracutaneously sensitized and the decreased reactions of the bacterially immune groups as compared with those of the normal animals are clearly apparent and were expected since these contrasting reactivities have become familiar through the studies of Swift and his coworkers. The P-immune animals likewise gave lesions smaller than the normal.

The results in the B.E.-immune groups of the three experiments were conflicting. In Experiments 1 and 2 (not shown in chart) the lesions were almost as small as those of the animals immunized intravenously with intact cocci, but in Experiment 3 (as shown in Chart 1A) they were larger than normal. No explanation was found for this unexpected reaction in the four rabbits of Experiment 3.

*Skin Tests with P.*—The skin reactions induced by intradermal injections of P in the rabbits of Experiment 3 are shown in Chart 1B. The enormous lesions of the intracutaneously sensitized animals are striking when compared with those of the normal controls. The average lesions of the intact coccus-immune group were similar in size to those of the normal animals, while those of B.E.-immune animals were smaller and very much more transient. The P-immune rabbits showed an immediate response more than twice as great as that of the normal animals, although less than that of the intracutaneously sensitized rabbits. The lesions in these two groups of animals illustrated well the differences between dermal reactivity of the anaphylactic and of the tuberculin types. Those of the former group had reached their maximal size by the end of 24 hours, were chiefly edematous in character, and subsided rapidly; in contrast, those of the intracutaneously sensitized group reached their maximal size only after 48 to 72 hours, were indurated as well as edematous, and subsided slowly.

In three of the seven normal rabbits the lesions induced by test injections of P showed a secondary increase in size about 4 days after their first appearance. This phenomenon was similar to the "secondary reaction" which occurs in normal rabbits injected intradermally with living bacterial cultures (2) except that it appeared several days earlier; and in this regard it should be noted that Schultz and Swift (12)

have observed the same early type of secondary reaction in normal rabbits injected with small amounts of horse serum.

*Skin Tests with S.*—Chart 1D records the results obtained when 0.25 mg. of S was injected intradermally into the rabbits of Experiment 3. Again, the largest lesions were shown by the bacterially hypersensitive group and the peak of the reaction was reached in 48 hours. Although smaller than the above, the lesions induced in the rabbits immunized with living culture were striking when compared with those of the normal controls. At the end of 24 hours these lesions were pink, edematous areas almost 25 times as voluminous as those of the normal rabbits, but they then rapidly decreased, showing the same size as those of the normal animals after 48 hours. The B.E.-immune animals showed essentially negative reactions, while those of the P-immune group became negative after 48 hours in spite of an initial response slightly greater than normal.

When a skin test dose of S ten times as large (2.5 mg.) was used, the results (Chart 1C) were essentially an exaggeration of those just described except in the case of the normal control group. The latter animals showed the expected small lesions after 24 hours; but a very pronounced increase in size then occurred, and reached its peak at 72 hours. The character of the lesions and the early occurrence of the enlargement make it improbable that it represented a secondary reaction of the hypersensitive type, but rather that the increase in size was due to some irritant in S solutions of this strength. This view is supported by the observation that similar effects are obtained in normal rabbits following intradermal injection of xylol in high concentration but not in low concentration (13). Furthermore, it is interesting to note that the irritant was probably of specific antigenic nature because the animals immunized with P and with B.E. were completely protected against this later reaction. So also were the rabbits immunized with living culture, in spite of the fact that they showed initial responses almost five times as great as those of the normal controls.

#### DISCUSSION

The differences between the reactivity to tuberculin of guinea pigs immunized with tuberculo-proteins and those inoculated with tubercle bacilli have long been known through the studies of Baldwin (14),

Krause (15), Zinsser (16), Dienes and Mallory (17), and many others. It is accepted that reactivity of the sort first mentioned is dependent upon an antigen-antibody reaction of the classical anaphylactic or Arthus type, but that true tuberculin hypersensitivity is independent of obviously demonstrable antibodies; and it is probable that these reactivities represent phenomena which can be elicited by bacteria in general. Various aspects of immunization and sensitization with streptococci and their fractions have been reported by a number of investigators, and it has been clearly shown that treatment with the various soluble fractions leads to the anaphylactic type of response on subsequent injection (18, 19), but that suitable intracutaneous inoculation with intact cocci induces sensitivity of the tuberculin type (3). In the work here reported, analogies to the studies with the tubercle bacillus are obvious.

For purposes of comparison, the results of serologic and dermal tests have been shown diagrammatically in Chart 2. As expected, there was a certain degree of correspondence. Thus, the animals immunized with P gave high anti-P precipitin titers and reacted with large lesions when cutaneously tested with P. These lesions reached their peaks comparatively early, were largely edematous in character, and subsided rapidly, as indicated by the sloping line in Chart 2. Similar lesions were induced by intradermal injections of S into the rabbits having high anti-S precipitin titers. As has been noted by Francis and Tillett (20), these probably represent carbohydrate antigen-antibody reactions wholly comparable to the familiar Arthus phenomenon obtained with proteins. That the correspondence between precipitin titer and dermal reaction was not complete, however, is indicated by the responses of the rabbits immunized with B.E. This group showed skin lesions even smaller than those of normal controls when injected intradermally with P, in spite of the presence of circulating anti-P precipitins in titer as high as that of the P-immune group.

Worthy of comment also was the limited antigenic capacity of the bacterial emulsion. Immunization of rabbits by intravenous injections of living, formalinized or heat-killed cultures led to the production of agglutinins and type-specific precipitins in high titer and of anti-P precipitins in varying amounts. Similar immunization using the

bacterial emulsion, in which the cocci had been killed by mechanical grinding, induced no demonstrable antibodies until relatively massive doses had been injected; and then only anti-P precipitins and agglutinins of only low titer were found, while anti-S precipitins were doubtful. In other words, the ground bacteria acted antigenically like the extracted nucleoprotein fraction. A possible ex-

| Cutaneous reaction in rabbits        | Normal Rabbits |   |   | Intravenously immunized with intact cocci |   |   | Intravenously immunized with P |   |   | Intravenously immunized with B. E. |   |   | Intracutaneously sensitized with intact cocci |   |    |
|--------------------------------------|----------------|---|---|---|---|---|--------------------------------|---|---|------------------------------------|---|---|---|---|----|
|                                      | Intact cocci   | P | S | Intact cocci                              | P | S | Intact cocci                   | P | S | Intact cocci                       | P | S | Intact cocci                                  | P | S* |
| Relative size of cutaneous reactions | +++            |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
|                                      | ++             |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
|                                      | +              |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
|                                      | -              |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
|                                      | +              |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
| Relative titer of antibodies         | +++            |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
|                                      | ++             |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
|                                      | +              |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
|                                      | -              |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |
|                                      | +              |   |   |   |   |   |                                |   |   |                                    |   |   |   |   |    |

CHART 2. Schematic comparison of cutaneous and serologic reactions of rabbits treated in different ways.

Neg. = negative reaction.

\*S extract possibly contained traces of P.

planation for this (21) is that the type-specific fraction of the antigenic complex was split off during the grinding, leaving S merely as haptene and P as the only effective antigen. Although the mechanism underlying this change is not yet clear and is still under investigation, the fact that pulverized streptococci had such limited antigenic capacity would lead one to question the validity of Krueger's suggestion (22) that bacterial antigens be prepared by mechanical

grinding in order to obtain more efficient immunizing agents. From the results described here it would seem that, at least in the case of the indifferent streptococcus, the mechanically ground antigen is efficient in calling forth non-type-specific antibodies, but is of very little value where type-specific immunization is desired.

Distinct from the findings in the immune groups were those of the intracutaneously sensitized rabbits. Of all the groups, these animals showed by far the largest lesions to each of the four skin test substances used although their sera gave negative precipitin tests; and these dermal responses were delayed in reaching their peaks and persisted as large indurated nodules for many days. Whatever the mechanism underlying the exaggerated dermal reactions of intracutaneously sensitized animals may eventually prove to be, it is clear that, with the streptococcus as well as with the tubercle bacillus, the local reactions are not dependent upon the presence of demonstrable circulating precipitins of either the species- or type-specific varieties.

A difference between the present study and those dealing with tubercle bacilli exists in the apparent ability of S to call forth the tuberculin type of reaction in the intracutaneously sensitized animals. Zinsser at first (23) observed typical reactions following the intradermal injection into tuberculous guinea pigs of both protein and carbohydrate ("residue") fractions of the tubercle bacillus, but later (24), using more highly purified materials, found only the protein capable of inducing the reaction. Similarly, Laidlaw and Dudley (25), Long and Seibert (26), Mueller (27), Dorset, Henley and Moskey (28), and Dienes (29) obtained local responses following intradermal injection of tuberculo-protein into tuberculous guinea pigs but did not observe them following similar injections of tuberculo-carbohydrate.<sup>2</sup> Only Petroff (30) found tuberculo-carbohydrate active in the tuberculin sense, and it is interesting to note that the animals which he used were rabbits rather than guinea pigs. In the present study, the streptococcal carbohydrate used was highly potent in inducing dermal reactions in rabbits intracutaneously sensitized with the homologous streptococcus. Whether this represents a difference between the skin reactivity of rabbits and guinea pigs, or whether bacterial sensitivity

<sup>2</sup> Not all these authors called their material carbohydrate but, from the methods of preparation, it may be assumed that all were dealing with essentially the same substances.

to streptococci differs from that to tubercle bacilli in this regard, cannot be stated. Another possible explanation is that the carbohydrate extract employed by Petroff and that used in the present study, contained enough contaminating nucleoprotein to give rise to reactions in bacterially sensitized animals. Against this explanation, however, is the fact that the S extract used in Experiment 3 had been subjected to ultrafiltration through a collodion membrane and contained only doubtful traces of P, for the serum of only one P-immune rabbit gave precipitation with S extract and this reaction was merely a questionable one in the lowest dilution (see Table V). Actually the lesions resulting from the injection of 0.25 mg. of S into intracutaneously sensitized rabbits were comparable in size to those induced by 0.1 mg. of P, and it seems hardly possible that traces of P in the S extract could have approached this amount. Nevertheless, this remains an important possibility, and experiments are in progress to check the results with even more highly purified S.

There appear to be three possible explanations of the wide hypersensitivity of the intracutaneously sensitized rabbits to all the test substances used: (a) that the skin responses are due to an antigen-antibody reaction involving a fraction common to all the test substances, (b) that they represent antigen-antibody reactions to each of various fractions used in testing, or (c) that they are due to a general heightening of tissue reactivity independent of antigen-antibody interaction and hence are non-specific in nature. Further studies are in progress to test these possibilities.

#### SUMMARY AND CONCLUSIONS

Rabbits were immunized intravenously with intact indifferent streptococci, with homologous P fraction, and with an emulsion of mechanically ground cocci; others were sensitized by intravenous injection of the intact microorganisms. Their serologic and dermal reactions to these materials and to the homologous S fraction were compared with those of normal animals. The dissociation, in certain instances, between circulating antibody and dermal reactivity was noteworthy. From the results the following conclusions were drawn.

1. Intradermal injection of a soluble streptococcal protein into a rabbit immunized intravenously with that protein leads to the immediate anaphylactic type of skin response; while similar dermal testing



grinding in order to obtain more efficient immunizing agents. From the results described here it would seem that, at least in the case of the indifferent streptococcus, the mechanically ground antigen is efficient in calling forth non-type-specific antibodies, but is of very little value where type-specific immunization is desired.

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1. Intradermal injection of a soluble streptococcal protein into a rabbit immunized intravenously with that protein leads to the immediate anaphylactic type of skin response; while similar dermal testing

of a rabbit sensitized by intracutaneous inoculation of the intact microorganism induces the delayed (tuberculin) type of response.

2. The induction of the immediate type of dermal reaction to streptococcal protein requires more than the mere presence of a high serum precipitin titer to that protein.

3. Lesions of the immediate type can be induced by the intradermal injection of a streptococcal carbohydrate into rabbits immunized intravenously with intact cocci and showing a high serum precipitin titer to that carbohydrate.

4. Intravenous immunization of rabbits with an emulsion of mechanically ground indifferent streptococci leads to the production of only non-type-specific antibodies.

5. It is possible that carbohydrate as well as protein fractions of indifferent streptococci are capable of eliciting the delayed type of dermal response in rabbits intracutaneously sensitized with that microorganism.

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# DISTRIBUTION OF BLOOD GROUP PROPERTIES AND BLOOD GROUP PROPERTY DESTROYING FACTORS IN THE INTESTINAL TRACT OF MAN

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Investigations carried out during recent years have shown that the biological characteristics known since Landsteiner's discovery as blood groups concern the entire organism and not only the blood. Group specific substances have been found in alcoholic and in watery extracts of organs of man and animals (1-3). The quantitative distribution of group properties, however, is quite different in various organs (4). Especially rich in group substances are, for instance, kidneys, lungs, pancreas, and the mucous membranes of the intestinal tract; on the other hand the brain contains only traces of the substances, if any. Moreover, they are found not only in the tissues of the organism, but also in its secretions. Here also we encounter great differences in the concentration of group properties. Thus the spinal fluid is almost free of group substances, while saliva and stomach juice generally are rich in them. They are constantly excreted with the urine. Hence, continuous regeneration of group substances must take place.

In view of the high content of the stomach juice in group specific substances it has been surprising to find that the feces are lacking in these properties.<sup>1</sup> Schiff and coworkers (7, 8) showed that aqueous extracts of feces (as well as saliva) destroy the group specific properties. It is possible to obtain Berkefeld filtrates of feces which also destroy the group specific substances (9, 10). The activity of feces extracts toward the group specific properties is attributed by Schiff and his coworkers to the presence of a specific blood group property destroying enzyme which is produced by the body itself. Many facts, however, seem to speak against

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<sup>1</sup> This statement is not affected by the fact that small amounts of group specific substances can be demonstrated in feces by means of special methods as described recently by Hodyo (5) and Moharrem (6).

the assumption that the blood group destroying agent is a secretion product of the body such as pepsin or trypsin. Witebsky and Satoh found that an increase of the blood group property destroying agent obtained from saliva takes place in progressive transplantation (chain experiments) in which a mixture of serum and egg white was used as medium. An increase up to 2,000 times the original amount of the blood group destroying factors was found. In corroboration of these findings Sievers (11, 12) reported an increase in effectiveness of the blood group property destroying agent up to 1 million of the original strength. Sievers used broth as the medium. Although we cannot make any definite statement concerning the origin of the blood group destroying agent we have to consider the possibility of its derivation from microorganisms or their growth products. Very recently the destruction of blood group specific substances by microorganisms has been described. A *Myxobacterium* isolated from vegetable sources by Morgan and Thyssen, was found effective against the group specific substance A by Landsteiner and Chase (13). Schiff (14) observed the destruction of blood group specific substances by *B. welchii*.

The following experiments deal with whether the agent destroying the blood group property occurs in the entire intestinal tract or only in parts of it. Inasmuch as there appears to be only a brief reference in the literature to the presence of blood group specific substance A in the content of the jejunum (7), an attempt has also been made by us to determine in which part of the intestinal tract the blood group properties themselves are present.

### *Material*

Extracts of intestinal content were prepared in the following way: One part of the material was mixed with 10 parts of physiological salt solution. When the specimens were solid the mixture of saline and feces was shaken thoroughly in a shaking apparatus for about 20 to 30 minutes and then centrifuged.

### *Method*

Several methods are available to determine the presence or absence of group properties. In the following experiments the so called hemolysis inhibition test was mostly used. This test is based on the fact that a group specific A-antiserum is rich in sheep cell hemolysins. By mixing dilutions of group specific A-antiserum with A containing material, the group specific antibodies are fixed and simultaneously the sheep cell hemolysins are bound. Hemolysis of subsequently added sheep cells is thus prevented.

To determine the presence of blood group destroying agent, its action toward peptone has been used. Witte peptone (1 per cent) known to contain A substance was prepared, heated in a water bath, filtered, and kept under sterile conditions.

*Experimental*

*Experiment 1. Quantitative Determination of Group Substance A in the Content of Various Parts of the Intestinal Tract.*—The experiment was set up as follows: Decreasing amounts (volume 0.2 cc.) of extracts<sup>2</sup> of the contents of (a) jejunum, (b) middle ileum, (c) lower ileum, (d) ascending colon, (e) descending colon were mixed with 0.2 cc. of a suitable dilution of a group specific A-antiserum. The mixtures were incubated at room temperature for 20 minutes. Then 0.2 cc. of a 5 per cent sheep cell suspension and 0.2 cc. of guinea pig serum in a dilution of 1

TABLE I

*Hemolysis of Sheep Cells by Complement and A-Antiserum after Treatment of the Latter with Intestinal Content Extracts*

| Amount of<br>extract of<br>intestinal<br>content | (a) Jejunum |         | (b) Middle<br>ileum |         | (c) Lower<br>ileum |         | (d) Ascending<br>colon |         | (e) Descending<br>colon |         |
|--|-------------|---------|---------------------|---------|--------------------|---------|------------------------|---------|-------------------------|---------|
|  | $\alpha$    | $\beta$ | $\alpha$            | $\beta$ | $\alpha$           | $\beta$ | $\alpha$               | $\beta$ | $\alpha$                | $\beta$ |
| 0.2  | 0           | 0       | 0                   | 0       | 0                  | 0       | 0                      | c.      | tr.                     | c.      |
| 0.04   | 0           | 0       | 0                   | 0       | 0                  | 0       | m.                     | c.      | ac.                     | c.      |
| 0.008  | 0           | 0       | 0                   | 0       | tr.                | c.      | ac.                    | c.      | c.                      | c.      |
| 0.0016   | 0           | 0       | 0                   | 0       | m.                 | c.      | c.                     | c.      | c.                      | c.      |
| 0.00032  | 0           | tr.     | 0                   | m.      | ac.                | c.      | c.                     | c.      | c.                      | c.      |
| 0.000064   | 0           | c.      | tr.                 | c.      | c.                 | c.      | c.                     | c.      | c.                      | c.      |
| 0.0000128  | ac.         | c.      | ac.                 | c.      | c.                 | c.      | c.                     | c.      | c.                      | c.      |
| 0.00000256                                       | ac.         | c.      | c.                  | c.      | c.                 | c.      | c.                     | c.      | c.                      | c.      |
| 0  | c.          | c.      | c.                  | c.      | c.                 | c.      | c.                     | c.      | c.                      | c.      |

$\alpha$  = hemolysis after 20 minutes.

$\beta$  = hemolysis after 40 minutes.

c. = complete hemolysis.

ac. = almost complete hemolysis.

m. = moderate hemolysis.

tr. = traces of hemolysis.

0 = no hemolysis.

to 10 were added. The resulting hemolysis was noted ( $\alpha$ ) after 20 minutes, ( $\beta$ ) after 2 hours, incubation at 37°C. (Table I).

As is seen from Table I, the contents of the jejunum as well as that of the middle ileum are rich in group specific substance A. The lower ileum contains only a moderate amount of A substance, while

<sup>2</sup> Before the experiment was set up the extracts were kept in a water bath at 65-70°C. for 10 minutes to counteract anticomplementary effects.



the colon contents are almost completely free. The experiment shows a steady decrease in A substance in the intestinal contents in adults, from above downwards.

Similar results were obtained in many cases (with a few exceptions which will be described below) quite independent of the disease. Thus the described distribution of the group specific A substance in the contents of the intestinal tract may be called a physiological one.

*Experiment 2. Quantitative Determination of Blood Group Property Destroying Factors in the Contents of Various Parts of the Intestinal Tract.*—For the quantita-

TABLE II

*Hemolysis of Sheep Cells by Complement and Group Specific A-Antiserum after Treatment of the Latter with a Mixture of Peptone and Intestinal Content Extracts*

| Amount of<br>peptone-extract<br>mixture | (a) Jejunum |         | (b) Terminal<br>ileum |         | (c) Cecum |         | (d) Sigmoid |         | (e) Peptone<br>control |         |
|---|-------------|---------|-----------------------|---------|-----------|---------|-------------|---------|------------------------|---------|
|   | $\alpha$    | $\beta$ | $\alpha$              | $\beta$ | $\alpha$  | $\beta$ | $\alpha$    | $\beta$ | $\alpha$               | $\beta$ |
| 0.25                                    | 0           | 0       | 0                     | c.      | c.        | c.      | c.          | c.      | 0                      | 0       |
| 0.125                                   | 0           | 0       | tr.                   | c.      | c.        | c.      | c.          | c.      | 0                      | 0       |
| 0.0625                                  | 0           | 0       | m.                    | c.      | c.        | c.      | c.          | c.      | 0                      | 0       |
| 0.03125                                 | 0           | 0       | ac.                   | c.      | c.        | c.      | c.          | c.      | 0                      | 0       |
| 0.0156                                  | 0           | tr.     | c.                    | c.      | c.        | c.      | c.          | c.      | tr.                    | m.      |
| 0.0078                                  | m.          | ac.     | c.                    | c.      | c.        | c.      | c.          | c.      | m.                     | ac.     |
| 0                                       | c.          | c.      | c.                    | c.      | c.        | c.      | c.          | c.      | c.                     | c.      |

$\alpha$  = hemolysis after 15 minutes.

$\beta$  = hemolysis after 2 hours.

tive determination of the blood group destroying agent its action upon Witte peptone was used in the following way: 1 cc. each of an extract of contents from the (a) jejunum, (b) terminal ileum, (c) cecum, (d) sigmoid, obtained from a person belonging to Group O as well as (e) saline control were mixed with 1 cc. of 1 per cent Witte peptone solution and incubated at 37°C. The resulting hemolysis is shown in Table II.

Table II shows that the mixture of peptone and jejunum contents reacts as strongly as the peptone itself upon the group specific A-antiserum. The mixture of peptone and terminal ileum content displays only a moderate inhibition of hemolysis. The cecum and sigmoid content-peptone mixtures proved absolutely negative. This

means that the blood group property destroying factors first appear in the terminal ileum and that their effectiveness increases in the cecum.<sup>3</sup>

In a few cases, however, intestinal content extracts from the lower part of the ileum displayed slight potency only, or even proved to be ineffective. In these cases the blood group destroying agent was found first in the content of the cecum and of the colon.

The comparison of the distribution of the A substance in the content of the intestinal tract (see Table I) with that of the blood group property destroying factors (see Table II) displays a close parallelism in the disappearance of the blood group substance on the one hand and the increase in blood group destroying agent on the other.

*Experiment 3. Simultaneous Presence of Blood Group Specific Substance A and Blood Group Destroying Factors within the Intestinal Tract.*

—The question arose whether a simultaneous presence of blood group substance and blood group destroying factors occurs anywhere in the intestinal tract. To elucidate this question blood group substance A as well as blood group destroying agent were determined in the various parts of the intestinal tract of a person belonging to Group A. The titration of the A substance was done as in Experiment 1, that of the blood group destroying agent as in Experiment 2. The results are given in Tables III and IV.

As in the case of the findings in Experiment 1, Table III shows that the contents of jejunum and ileum are rich in group specific A substance, while the cecum contains only moderate amounts and the content of the sigmoid is completely lacking in A substance.

Table IV shows that the extracts from the large intestine are not only free of A substance, but they even destroy the A substance of peptone. On the other hand the extracts of the jejunum and the ileum content do not exhibit any blood group destroying power. The cecum content extract, however, contains blood group destroying agent as well as blood group substance A, although the latter seems to be present in relatively small amounts only. This observation resembles Witebsky and Satoh's demonstration of the simultaneous existence of blood group substance and blood group destroying factors in the feces of rabbits.

<sup>3</sup> In all the experiments the group specific nature of the inhibition of the sheep cell hemolysis was controlled by simultaneous examination with sheep cell antiserum.

*Experiment 4. The Influence of Time on the Effectiveness of the Blood Group Property Destroying Factors.*—It is known from the study of the blood group property destroying agent that the time

TABLE III

*Hemolysis of Sheep Cells by Complement and Group Specific A-Antiserum after Treatment of the Latter with Intestinal Content Extract*

| Amount of intestinal content extract | (a) Upper jejunum |         | (b) Lower jejunum |         | (c) Ileum |         | (d) Cecum |         | (e) Sigmoid |         |
|--------------------------------------|-------------------|---------|-------------------|---------|-----------|---------|-----------|---------|-------------|---------|
|                                      | $\alpha$          | $\beta$ | $\alpha$          | $\beta$ | $\alpha$  | $\beta$ | $\alpha$  | $\beta$ | $\alpha$    | $\beta$ |
| 0.2                                  | 0                 | 0       | 0                 | 0       | 0         | 0       | 0         | m.      | c.          | c.      |
| 0.1                                  | 0                 | 0       | 0                 | 0       | 0         | 0       | tr.       | m.      | c.          | c.      |
| 0.05                                 | 0                 | 0       | 0                 | 0       | 0         | 0       | m.        | m.      | c.          | c.      |
| 0.025                                | 0                 | 0       | 0                 | 0       | 0         | 0       | m.        | ac.     | c.          | c.      |
| 0.0125                               | 0                 | 0       | 0                 | 0       | 0         | 0       | m.        | ac.     | c.          | c.      |
| 0.00625                              | 0                 | 0       | 0                 | 0       | 0         | 0       | m.        | c.      | c.          | c.      |
| 0                                    | c.                | c.      | c.                | c.      | c.        | c.      | c.        | c.      | c.          | c.      |

$\alpha$  = hemolysis after 20 minutes.

$\beta$  = hemolysis after 40 minutes.

TABLE IV

*Hemolysis of Sheep Cells by Complement and Group Specific A-Antiserum after Treatment of the Latter with a Mixture of Peptone and Intestinal Content Extract*

| Amount of peptone-extract mixture | (a) Upper jejunum |         | (b) Lower jejunum |         | (c) Ileum |         | (d) Cecum |         | (e) Sigmoid |         | (f) Peptone control |         |
|-----------------------------------|-------------------|---------|-------------------|---------|-----------|---------|-----------|---------|-------------|---------|---------------------|---------|
|                                   | $\alpha$          | $\beta$ | $\alpha$          | $\beta$ | $\alpha$  | $\beta$ | $\alpha$  | $\beta$ | $\alpha$    | $\beta$ | $\alpha$            | $\beta$ |
| 0.2                               | 0                 | 0       | 0                 | 0       | 0         | 0       | tr.       | m.      | m.          | c.      | 0                   | 0       |
| 0.1                               | 0                 | 0       | 0                 | 0       | 0         | 0       | m.        | ac.     | m.          | c.      | 0                   | 0       |
| 0.05                              | 0                 | 0       | 0                 | 0       | 0         | 0       | m.        | ac.     | m.          | c.      | 0                   | 0       |
| 0.025                             | 0                 | 0       | 0                 | 0       | 0         | 0       | ac.       | c.      | ac.         | c.      | 0                   | 0       |
| 0.0125                            | 0                 | 0       | 0                 | 0       | 0         | 0       | c.        | c.      | ac.         | c.      | m.                  | m.      |
| 0.00625                           | 0                 | 0       | 0                 | 0       | 0         | 0       | c.        | c.      | ac.         | c.      | ac.                 | c.      |
| 0                                 | c.                | c.      | c.                | c.      | c.        | c.      | c.        | c.      | c.          | c.      | c.                  | c.      |

$\alpha$  = hemolysis after 15 minutes.

$\beta$  = hemolysis after 40 minutes.

factor plays an important rôle in its effectiveness. Therefore, the question arose as to whether or not it is possible to demonstrate differences in the effectiveness of the blood group destroying factors by

shortening of the incubation period. The following experiment was set up.

Equal amounts of extracts of the content of (a) jejunum, (b) middle ileum, (c) terminal ileum, (d) colon, (e) physiological saline as control were mixed with Witte peptone (1 per cent solution) and incubated for (a) 3 hours at 37°C., (b) 15 hours at 37°C. The mixtures were then heated for 10 minutes in a water bath at 65–70°C. The titration of the A content of the various mixtures was done as in Experiment 2. The results are given in Table V.

TABLE V

*Hemolysis of Sheep Cells by Complement and Group Specific A-Antiserum after Treatment of the Latter with a Mixture of Peptone and Intestinal Content Extract*

| (a) Incubation time 3 hrs. at 37°C.     |             |         |                     |         |                       |         |           |         |                        |         |
|---|-------------|---------|---------------------|---------|-----------------------|---------|-----------|---------|------------------------|---------|
| Amount of<br>peptone-extract<br>mixture | (a) Jejunum |         | (b) Middle<br>ileum |         | (c) Terminal<br>ileum |         | (d) Colon |         | (e) Peptone<br>control |         |
|   | $\alpha$    | $\beta$ | $\alpha$            | $\beta$ | $\alpha$              | $\beta$ | $\alpha$  | $\beta$ | $\alpha$               | $\beta$ |
| 0.067                                   | 0           | 0       | 0                   | 0       | 0                     | c.      | c.        | c.      | 0                      | 0       |
| 0.0233                                  | 0           | 0       | 0                   | 0       | m.                    | c.      | c.        | c.      | 0                      | 0       |
| 0.0078                                  | m.          | c.      | m.                  | c.      | c.                    | c.      | c.        | c.      | m.                     | c.      |
| 0                                       | c.          | c.      | c.                  | c.      | c.                    | c.      | c.        | c.      | c.                     | c.      |
| (b) Incubation time 15 hrs. at 37°C.    |             |         |                     |         |                       |         |           |         |                        |         |
|   | $\alpha$    | $\beta$ | $\alpha$            | $\beta$ | $\alpha$              | $\beta$ | $\alpha$  | $\beta$ | $\alpha$               | $\beta$ |
| 0.067                                   | 0           | 0       | 0                   | 0       | ac.                   | c.      | c.        | c.      | 0                      | 0       |
| 0.0233                                  | 0           | 0       | 0                   | 0       | c.                    | c.      | c.        | c.      | 0                      | 0       |
| 0.0078                                  | m.          | c.      | m.                  | c.      | c.                    | c.      | c.        | c.      | m.                     | c.      |
| 0                                       | c.          | c.      | c.                  | c.      | c.                    | c.      | c.        | c.      | c.                     | c.      |

$\alpha$  = hemolysis after 20 minutes.

$\beta$  = hemolysis after 2 hours.

As is seen from Table V there is a marked difference in the effectiveness of the terminal ileum content extract depending upon the incubation period. After an incubation of 3 hours the A substance of the peptone is partly destroyed by the blood group destroying agent, while after 15 hours incubation the A quality has completely disappeared. The blood group destroying agent from the colon, however, destroys the entire A content of the peptone after 3 hours incubation. On the other hand, there is no trace of blood group property destroying activity to be found in the extracts of the jejunum and central ileum content, even after 15 hours incubation time.

It follows from the experiments mentioned above that there is no abrupt increase in the concentration of the blood group destroying agent in any given site of the intestinal tract. Its concentration increases rather gradually. It can be found first in low concentration in the lower part of the ileum and reaches its highest potency within the large intestine.

*Experiment 5. Distribution of Blood Group Property Destroying Factors in Cases with Intestinal Involvement.*—The above described characteristic distribution of the blood group destroying agent in the contents of the intestinal tract differed in a few cases with intestinal abnormalities. Two are of special interest.

The first was a carcinoma of the rectum with a colonic fistula, the second a stricture in the lower part of the colon due to lymphogranuloma. Intestinal contents from two tumor cases without involvement of intestine were used for control experiments, one was a case of chorionepithelioma; the other a case of tumor of the lungs. The experiment itself was carried out as described in Experiment 2. Specimens of the content of the following parts of the intestinal tract were examined: (a) jejunum, (b) upper ileum, (c) terminal ileum, (d) cecum. These extracts were mixed with equal amounts of 1 per cent Witte peptone solution and kept overnight in the incubator at 37°C. Then the mixtures were heated and the A content determined as described above. The results are given in Table VI.

Table VI shows that the content of the jejunum as well as of the ileum in two cases with intestinal involvement (colonic fistula and stricture due to lymphogranulomatosis, respectively) displayed a marked blood group destroying capacity. On the other hand the corresponding specimens of the tumor cases without intestinal involvement were completely ineffective.

#### DISCUSSION

The experiments reported in the present paper show that the contents of the upper intestinal tract (jejunum and upper ileum) of man belonging to Group A are usually rich in group specific substances.

The steady decrease in group specific substances in the intestinal tract in adults runs parallel to the increase in blood group property destroying power. Thus a link exists between the disappearance of group properties and the appearance of the blood group property destroying factors in the intestinal tract. There is a zone, however,

in which the effectiveness of the blood group destroying agent has not yet reached its complete power, so that a certain amount of group specific substance may be still present. This usually happens in the lower part of the ileum or in the cecum.

It is known that not all persons belonging to Group A excrete the group properties in their saliva. It is necessary therefore to find whether or not similar differences are encountered concerning the presence of group qualities in the content of the intestinal tract.

TABLE VI

*Hemolysis of Sheep Cells by Complement and Group Specific A-Antiserum after Treatment of the Latter with a Mixture of Peptone and Intestinal Content Extract*

| (a) Lymphogranulomatosis with stricture in rectum |             |         |                    |         |                       |         |           |         |                        |         |
|---|-------------|---------|--------------------|---------|-----------------------|---------|-----------|---------|------------------------|---------|
| Amount of<br>peptone-extract<br>mixture           | (a) Jejunum |         | (b) Upper<br>ileum |         | (c) Terminal<br>ileum |         | (d) Cecum |         | (e) Peptone<br>control |         |
|   | $\alpha$    | $\beta$ | $\alpha$           | $\beta$ | $\alpha$              | $\beta$ | $\alpha$  | $\beta$ | $\alpha$               | $\beta$ |
| 0.067   | m.          | c.      | m.                 | c.      | m.                    | c.      | m.        | c.      | 0                      | 0       |
| 0.0233  | ac.         | c.      | ac.                | c.      | ac.                   | c.      | ac.       | c.      | 0                      | 0       |
| 0.0078  | c.          | c.      | c.                 | c.      | c.                    | c.      | c.        | c.      | tr.                    | m.      |
| 0   | c.          | c.      | c.                 | c.      | c.                    | c.      | c.        | c.      | c.                     | c.      |

| (b) Rectum carcinoma-colon fistula |          |         |          |         |          |         |          |         |          |         |
|------------------------------------|----------|---------|----------|---------|----------|---------|----------|---------|----------|---------|
|                                    | $\alpha$ | $\beta$ | $\alpha$ | $\beta$ | $\alpha$ | $\beta$ | $\alpha$ | $\beta$ | $\alpha$ | $\beta$ |
| 0.067                              | m.       | ac.     | m.       | ac.     | m.       | c.      | m.       | c.      | 0        | 0       |
| 0.023                              | ac.      | c.      | ac.      | c.      | ac.      | c.      | ac.      | c.      | 0        | 0       |
| 0.0078                             | c.       | c.      | c.       | c.      | c.       | c.      | c.       | c.      | tr.      | m.      |
| 0                                  | c.       | c.      | c.       | c.      | c.       | c.      | c.       | c.      | c.       | c.      |

$\alpha$  = hemolysis after 15 minutes.

$\beta$  = hemolysis after 1 hour.

A marked change in the above described distribution of blood group substance and blood group destroying agent in the content of the intestinal tract was found in two cases with intestinal involvement. It is very likely, however, that the presence of the blood group destroying agent in the jejunum of these cases is not due to the actual disease but to mechanical obstruction. Further studies are necessary to elucidate the question as to whether there is a relation between an abnormal bacterial invasion of the upper intestinal tract and the appearance of blood group property destroying factors. Other cases

with intestinal diseases have not been available thus far. Special attention should be given to pernicious anemia and related forms of anemia.

## SUMMARY

1. The blood group specific substance A is present in the contents of the jejunum and upper ileum of persons belonging to Group A.
2. A marked decrease in the amount of A substance occurs in the lower ileum and cecum, while colon and rectum contents of adults are more or less free of group substance A.
3. The blood group destroying agent first appears in the lower ileum or cecum and its effectiveness increases in the lower parts of the large intestine.
4. In two cases with intestinal obstruction the blood group destroying agent could be demonstrated in the contents of the jejunum and ileum.

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# BILE FISTULAS AND RELATED ABNORMALITIES

## BLEEDING, OSTEOPOROSIS, CHOLELITHIASIS AND DUODENAL ULCERS

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During the past twenty years a good deal of work has been reported by Hooper, Foster (4), Smith (14), Hawkins (6), Whipple and associates dealing with the metabolism of bile pigments, bile salts and cholesterol. This work called for the use of several types of biliary fistula and observations were made first at the Hooper Foundation, the University of California, and later in Rochester. Gradually a good deal of information has accumulated relative to the proper care of the bile fistula animal but in spite of all precautions we observe all too frequently various types of abnormality which disturb the research program and nullify experimental observations. It is a commonplace to observe in the voluminous literature of this subject that investigators frequently will study the bile elimination in a bile fistula animal which shows a rapid loss of weight. Such observations are practically worthless as an animal of this sort is abnormal and would be found to be suffering from some of the abnormalities described below. Any value that attaches to such observations would then be related to that particular abnormal state but not to a normal base line.

Three types of bile fistula have been used in this series of observations.

1. The common type of open bile fistula used by practically all of the older workers is unsatisfactory in many respects but observations of value have been made (4). This fistula is always infected but usually by non-pathogenic bacteria, and these dogs if draining well and properly fed will continue in good health and weight equilibrium for many months. It was observed that liver feeding at intervals would do much to prevent intoxication and in particular to prevent de-



calcification of the skeleton described below. A modification of this open fistula developed occasionally in some dogs,—that is a tiny fistulous tract along the gap in the common bile duct which permitted some bile to flow into the duodenum during the night when the outer fistula was not draining freely because of removal of the collection tube. Such bile fistula dogs remained in perfect health for many years and were valuable experimental animals. These dogs emphasized the importance for normal function and health of a small amount of bile flowing daily into the duodenum.

2. The popular closed sterile bile fistula as devised by Rous and McMaster (12) is a much more satisfactory type of fistula but it too has many drawbacks. Precipitates and calculi will form in the rubber tube in spite of sterile conditions and these dogs rarely can be utilized longer than 4–6 months but they are admirable for certain experiments and do away with ordinary types of infection.

3. A very useful type of bile fistula was devised by Kapsinow, Engle and Harvey and utilizes the renal pelvis for the fistulous tract (7). The bile can be collected in the urine without difficulty and the tract usually remains sterile. Such dogs have been under observation in our laboratory in perfect health for many years but care must be given to the diet and some bile must be fed at intervals to avoid some of the intoxications described below. Calculi on occasion will develop in the renal pelvis and cause much trouble. With present methods of analysis this type of fistula cannot be used to study bile salt output as the mixture with urine introduces too many confusing additive factors.

In 1922 it was concluded after a general review of the subject (17) that “bile secretion into the intestine is necessary for normal health and even for actual continuation of life beyond a few months’ period.” We have found no experimental data which have modified this conclusion. Intoxications and abnormalities as they appear all too frequently in the régime of the bile fistula dog are viewed with disfavor by the investigator as they may interrupt or terminate the period of observation. Recently, however, we have become interested in certain abnormalities which can be produced experimentally, with intent to study the relief of the given abnormality by the administration of bile or some bile constituent.

#### *Abnormalities Related to Exclusion of Bile from the Intestine*

1. Acute intestinal disturbances with rapid weight loss and even death.
2. Purpuric tendency with spontaneous bleeding.
3. General osteoporosis with multiple spontaneous fractures.
4. Cholelithiasis with obstruction in the fistulous tract.

5. Duodenal ulcers with lack of appetite and hemorrhage or even perforation.

We wish to expand somewhat this brief classification.

1. *Acute Intestinal Disturbances*.—This abnormality is common in the reports in the general literature and is usually associated with rapid weight loss. A diet poor in fat and animal proteins will be helpful and as little as 50 cc. dog bile daily will return the dog to normal. We found also that whole liver feeding would usually prevent this disturbance and occasional liver feeding periods would serve to keep the dog in a healthy condition even with an open infected bile fistula. The renal type of fistula can be kept in perfect clinical condition and weight balance on a salmon bread diet plus 50 cc. dog bile daily. The preparation and constituents of the salmon bread have been carefully described (18). It contains wheat flour, bran, potato starch, canned salmon, sugar, cod liver oil, canned tomatoes, yeast and a salt mixture. The bread contains 10 per cent protein, 6.5 per cent fat and 83.4 per cent carbohydrate. When a bile fistula dog is being kept in an anemic state by bleeding it is necessary to add more than 50 cc. of bile daily to maintain health and prevent intoxication.

2. *Purpura and Spontaneous Bleeding*.—We are not aware that this complication in bile fistulas has been given much if any attention. It develops usually within 3–4 months when the bile is completely absent from the intestinal tract but purpura does not develop if obstructive jaundice is present in these dogs even when all bile is excluded from the intestine. It would seem that something is lost through the bile fistula which after 3 or more months causes abnormality of blood clotting. With obstructive jaundice this unknown factor is retained in the blood and coagulation is normal. Transfusion will cause cessation of bleeding but has no lasting effect as the abnormal state of blood clotting still persists, and hemorrhage may soon occur again. It seems probable that there is a deficiency of prothrombin in the circulating blood which at first causes delayed clotting time and finally spontaneous bleeding from the intestinal tract and other points of slight trauma. A detailed report will soon be published.

3. *General Osteoporosis with Spontaneous Fractures*.—It was observed that liver feeding in bile fistula dogs prevented this osteoporosis

although the mechanism of this reaction is not clear (17). Obstructive jaundice has no effect upon this condition and dogs with bile excluded from the intestine will develop osteoporosis whether there is any obstruction or not. It takes 10-12 months for this condition to develop, as the mineral reserve store is considerable and a negative balance must continue a long time before the ribs are thinned sufficiently to break or bend under normal conditions of exercise. The gross picture shows numerous greenstick fractures of the ribs and long bones with very thin shafts, so much so that these bones and the vertebrae at autopsy can be cut with a knife. Histologically the picture is that of human osteoporosis and need not be described in detail.

Pavlov (10) first indicated that exclusion of bile from the intestinal tract resulted in osteoporosis. Seifert (13) suggested the possibility that with no bile in the intestine vitamin D would not be absorbed and this would lead to a negative calcium and phosphorus balance. This hypothesis was supported by the studies of Tammann (15) since he found parenteral administration of vitamin D was beneficial. Greaves and Schmidt (2) have demonstrated a negative calcium and phosphorus balance in dogs and rats deprived of bile, and attribute it to a lack of absorption of vitamin D from the intestine. This negative balance can be changed to a positive one by subcutaneous administration of viosterol. In rats when desoxycholic acid and viosterol were mixed in the diet a positive balance was established whereas in the absence of the bile acid the balance remained in the negative phase.

4. *Cholelithiasis* is very rare in dogs and it has been shown that human gall stones placed in the dog's normal gall bladder will be slowly dissolved and vanish, but if the gall bladder shows inflammation the inserted gall stones remain undissolved (5, 1). However in the closed sterile fistula deposits are often formed in the rubber tube and may obstruct the lumen completely. This material may be various mixtures of mucus, cholesterol, bile pigment or salt. In the renal fistula however we have observed calculi with some frequency.

*Renal Fistula Dogs* (26 in number) have been studied for periods of more than 5 months, and gall stones have formed in six within the fistula tract and these have caused at times partial or practically

complete obstruction. In two male dogs, the stones lodged in the urethra and were removed by instrumentation. One female passed several stones. Indications of stones are jaundiced plasma, bloody urine and difficulty in the passing of urine. In one dog at autopsy the gall bladder was filled with numerous small stones. Analysis shows that the concretions are formed largely of cholesterol and pigment so that they are not renal or urinary bladder stones.

5. *Duodenal Ulcers* are also relatively common in all types of bile fistula dogs and this complication must be kept in mind. These ulcers may also be observed even more frequently with simple biliary obstruction.

*Duodenal Ulcers* have been found in nine out of a total of thirty-three dogs that lived more than 3 months after operation. One died as the result of massive intestinal hemorrhage; another with peritonitis as the result of perforation of an ulcer. A third dog which was a combination renal fistula and Eck fistula had a duodenal ulcer with adhesions between the left lobe of the liver and the wall of the intestine. Round worms had passed through the ulcer into the liver and from it into the peritoneal cavity with resulting peritonitis. The other five ulcers had caused no clinical disturbance. Three dogs had had the common bile duct surgically ligated and of these two had duodenal ulcers at autopsy.

### *Methods*

The various operations as well as postoperative and routine care of these bile fistula dogs have been carefully described in various publications. All operations were done under ether anesthesia. The clinical condition of these fistula dogs and the body weight must be carefully followed.

The stock kennel ration is made up of mixed hospital table scraps and contains much bread, potato and vegetables, a little meat and bones with variable amounts of butter.

The hemoglobin values are established from a fixed base line where 100 per cent = 13.8 gm. hemoglobin.

Clotting time has been determined by observing whole blood and by the study of recalcified plasma. It has been customary to combine 0.5 cc. of the plasma with 0.5 cc. of a 0.6 per cent solution of calcium chloride. This mixture was read at room temperature in small test tubes. The length of time necessary for a solid clot to form so that the tube can be inverted without dislodgement of the clot has been considered as the clotting time. Normal dog plasma has been used as a comparative control.

## EXPERIMENTAL OBSERVATIONS

A few of the most interesting dogs will be described below to illustrate the various abnormalities classified above. As the pictures of purpura or osteoporosis are so uniform it is not necessary to describe a large number of these animals.

*Purpura.*—When a closed sterile bile fistula or a sterile renal fistula dog is deprived of all bile by mouth and fed the standard salmon bread ration, the animal will remain in health for about 3–4 months. The dog then will show some delay in clotting time and finally spontaneous bleeding from gums, gastro-intestinal tract or venous punctures. Transfusions are beneficial at this stage and 50–100 cc. of normal dog blood in many instances will stop the bleeding and give a normal clotting reaction. This will be only a temporary relief but in an emergency the transfusion is invaluable and may save the life of a valuable animal. At this point permanent return to normal can be effected by feeding whole bile in large quantities—for example 100 cc. whole dog bile for a week interval.

The following histories are typical and illustrate the points noted above.

*Dog 30-147, Renal Fistula, Spontaneous Bleeding.*—The dog was operated upon under ether anesthesia on Feb. 5, 1931, and a gall bladder renal fistula made. On Feb. 9, dog bile (50 cc.) was given by stomach tube and continued daily until Apr. 10. On this date urobilin was found in the urine, indicating that there was infection somewhere in the fistula tract which is a most unusual occurrence in this type of fistula. Dog bile (200 cc.) was given for 8 days by stomach tube to cause a copious flow of bile which would flush out the fistula tract and possibly result in a clearing of the infection. No such favorable result was obtained so the dog was given a daily diet of kennel ration and all bile by mouth discontinued after Apr. 20. The dog continued to eat its food and the clinical condition remained excellent except that the hair became rough and felt sticky. On June 18, after a jugular vein puncture, bleeding occurred and on the following morning some blood was found beneath the cage. No hematoma could be demonstrated in the neck, the bleeding had ceased, and there was no recurrence of it until July 24, following another vena puncture. During the night there was bleeding from the puncture wound and in the morning the dog was found lying in the cage and was inactive in contrast to the usual liveliness displayed upon receiving attention. There was a diffuse bogginess in the tissues of the neck but no actual hematoma could be felt. Bleeding had apparently ceased. The dog remained inactive for the next 2 days and on the last day of life, July 26, breathing was labored.

It is of interest that on the day of operation, the dog weighed 13.5 kilos and after 3 months of complete bile deprivation on a kennel diet ration weighed 13.1 kilos. No indication of anemia was ever observed. The hemoglobin on Feb. 26, 3 weeks after operation, was 114 per cent and on July 16, 1931, was 102 per cent. Death occurred 3 months after bile deprivation began.

*Autopsy Findings.*—The mucous membranes of the mouth are pale. The subcutaneous tissues of the neck are boggy due to edema, but deeper in the muscles and in the tissues surrounding the trachea there is much hemorrhage extending from the larynx into the mediastinal tissues to the hila and beneath the pleura of the lungs and along the aorta to the diaphragm. The heart grossly shows areas of hemorrhage in the fat about the auricles and upon being opened subendocardial hemorrhage is found in the left ventricle. Blood from heart clotted in glass container only after 1½ hours. Valves and myocardium appear normal. Histological sections show normal muscle but there are areas of hemorrhage subendocardially and in the epicardial fat. Polymorphonuclear leucocytes are found near the extravasated blood in the epicardial fat. Sections of the aorta show adventitial hemorrhage.

The right lung grossly presents extravasated blood beneath the pleura at the hilum with pleural ecchymosis scattered elsewhere. Most of the lung is crepitant and normal except for a few small, firm areas which are bright red in color. The left lung is solid dark purplish-red and on section it is very wet with much blood in the fluid. There are areas of consolidation suggesting pneumonia. Histological sections show extravasated red cells about the bronchi and in the alveoli with edema. Inflammatory exudate is present in some areas. The spleen is small, mottled with red areas. In the section phagocytic cells containing iron pigment are conspicuous.

In the gross the liver appears normal but histologically in one section the bile ducts are dilated, full of polymorphs and there is some fibrosis of the surrounding tissues with infiltration of polymorphs and mononuclears. Some of the bile canaliculi contain brown colloid material. The fundus of the gall bladder is firmly healed into the pelvis of the right kidney and grossly appears normal. In sections there are a few round cells in the mucosa. The extrahepatic ducts are slightly dilated and the common bile duct is ligated and its continuity interrupted.

The right kidney shows a dilated pelvis with scarring and atrophy of the parenchyma as the result of operation. The left kidney is large but normal in appearance. Histologically the right kidney is scarred with atrophic, hyalinized glomeruli and collapsed tubules. Round cells are present in the increased connective tissue. Section from the left kidney shows normal structures. The ureters and bladder are grossly normal but in sections there are a few scattered round cells beneath the epithelium. In the colon there is blood adherent to the mucosa but the feces do not seem to contain it and are light clay colored. No bleeding points can be seen, and in the sections no abnormalities are found.

The bones are firm and show no evidence of decalcification. There is no abnormality of the bone marrow in histological sections.

*Dog 30-352, Renal Fistula, Very Little Bile Feeding, Fatal Spontaneous Bleeding.*—A gall bladder renal fistula was made on Aug. 12, 1931. For 12 days after operation the dog was fed kennel diet and then the diet was changed to 600 gm. salmon bread, 100 gm. salmon, and 100 gm. Klim mixed into a mash with water. No bile was given. The dog ate its food and remained in excellent condition until Dec. 20 when a large hematoma formed in the neck following vena puncture. It became inactive, coughed at times and refused to eat. On Dec. 21 ox bile 50 cc. was given by stomach tube and this was continued until Jan. 2, 1932. On Dec. 21 the blood took 23 minutes to clot into a solid jell. The hematoma gradually increased in size and on Dec. 23 whole blood (135 cc.) was transfused, and this stopped the bleeding. The dog on the next day appeared more normal and ate all its food. As the result of the hematoma being resorbed the output of bile pigment for the next 14 days was about twice the normal amount. On Dec. 31 whole blood clotted quickly and firmly. From Jan. 2 to May 14 all bile was discontinued, and the dog received the salmon bread diet but from Jan. 28 to Mar. 3 an egg yolk was added in order to learn whether cholesterol might influence clotting. During this period the blood clotted normally, the time varying from 4 to 8 minutes. On May 12 the clotting time was prolonged to 15 minutes and on May 14 the dog again became inactive although it ate its food. No hematoma was noted in the neck. May 16 the dog died.

Throughout this period of 9 months bile had been given on only 11 days and yet the dog never showed any anemia and had gained 3.5 kilos.

*Autopsy* discloses the presence of hemorrhage into the subcutaneous tissues and muscles on the left side of the neck, into the upper mediastinum, in the axillae, over belly and over the back. Blood is found about the abdominal aorta, in pelvic tissues and in the retroperitoneal tissue about the right kidney. The blood within the heart and vessels is fluid and rather thin. The viscera appear normal. The fistula tract is patent, and no communication is found between the duodenum and the ligated and severed common bile duct. The bones are firm and show no fractures.

*Dog 33-90, Renal Fistula, Purpura, Saved by Transfusions and Whole Bile Feeding.*—A gall bladder renal fistula was made on Mar. 13, 1934, and on Mar. 17 the dog weighed 18.6 kilos and had a hemoglobin level of 122 per cent. It was fed 600 gm. of kennel diet with no bile by mouth until Apr. 10 when 1 gm. of sodium taurocholate was added to the food. On April 23 the bile salt was increased to 3 gm. daily and on May 7 in addition to this three egg yolks were added. From May 20 to June 7 the diet was 600 gm. kennel diet only but on the latter date it was changed to 200 gm. cooked pig liver and 200 gm. of salmon bread and this was fed until June 25. On July 5 the hemoglobin was 80 per cent and this fall is due probably to the fact that 10 cc. of blood had been removed daily for blood cholesterol determinations. From July 1 to Oct. 3 the diet was 700 gm. kennel diet but 50 cc. ox bile was given by stomach tube commencing July 24. From Sept. 3 to 14 the dog was ill, left food with the weight dropping to 14.8 kilos. The

cause of this illness was not determined. Oct. 3 the diet was changed to 400 gm. salmon bread, 75 gm. salmon and 30 gm. Klim with 50 cc. ox bile by stomach tube. On Nov. 15 ferric ammonium citrate 25 cc. of a 1 per cent solution was added to the diet in an attempt to raise the hemoglobin from the level of 80 per cent. On Dec. 12 pig liver 100 gm. was added to the above diet and on Dec. 20 the liver was increased to 300 gm. This diet was fed until Jan. 15, 1935. The hemoglobin had not increased by all the iron and liver, but the weight was now 20.2 kilos and the clinical condition was excellent. Jan. 9 whole blood and recalcified plasma clotted in 13-15 minutes. Jan. 15 the diet was changed to 400 gm. salmon bread, 75 gm. salmon, 30 gm. Klim plus 1 gm. of sodium taurocholate with no bile by mouth. Jan. 18 there was blood in the urine and for the next 5 days much blood was passed with the urine. Jan. 22 the dog's pulse was rapid, respirations were quick, and it was thought best to transfuse 175 cc. whole blood in order to stop the bleeding. Jan. 24 the urine was free of blood. On day of transfusion whole blood clotted very slowly as in 20 minutes only a fragile clot had formed, but at the end of 40 minutes there was a solid clot which later retracted well. The day following transfusion it took 30 minutes for a fragile clot to form and this delay in clotting continued. Jan. 21 sodium taurocholate 1 gm. and 1 egg yolk were added to the salmon bread diet to see if these substances would influence clotting. On Feb. 5 dog bile 50 cc. was substituted because blood was passed in the urine Feb. 4, and on the next day blood oozed all day from a vena puncture wound. The vein was punctured at 9 a.m. and at 11:30 p.m. there was still oozing of blood and a small hematoma was present in the neck. Whole blood 140 cc. was transfused and shortly thereafter the oozing ceased, and in the morning there was no indication that any further bleeding had occurred. Recalcified plasma clotted in 25 minutes. Feb. 9 the dog bile was increased to 100 cc. daily, and on Feb. 20 recalcified plasma clotted in 9 minutes as contrasted with 6 minutes for normal dog plasma. Feb. 23 the dog bile was discontinued and 1 gm. sodium taurocholate was added to the food. 3 days later blood was present in the urine and continued to be present on succeeding days with large amounts of it on Mar. 2. On this day recalcified plasma clotted solid after 26 minutes. Mar. 3 the dog was found lying quietly in the cage whereas the day before it had been very active. Respirations were labored and a red cell hematocrit was only 16 per cent. Whole blood 220 cc. was transfused without any obvious improvement resulting so 2 hours later 270 cc. more blood was given. Respirations still remained labored in spite of all the blood and in the evening the dog was found dead.

*Autopsy* was performed immediately and the essential findings are given. In the anterior mediastinum there is a large mass which is composed of fat infiltrated with blood much of which is still fluid, and in both pleural cavities there is fluid blood, 750 cc. in amount with the lungs greatly compressed. No hematoma is found in the neck in relation to the jugular veins, so the bleeding was spontaneous and not related to jugular punctures. When the heart is opened well formed clots are found within the chambers.



All the viscera are normal grossly and histologically except for the atelectasis of the lungs. The gall bladder renal fistula is patent and the common bile duct ligated and severed and no communication is found between it and the duodenum. No bleeding point can be found in the urinary tract to explain the blood in the urine. The bones show no evidence of decalcification but the marrow is hyperplastic.

The important facts of this history are that ox bile and sodium taurocholate did not prevent the abnormality in blood clotting from developing. Whole bile caused a return to normal clotting. Beneficial effects of whole blood transfusion are strikingly demonstrated by the autopsy. The blood in the mediastinal fat and pleural cavities was unclotted and even after standing in a flask for 24 hours only a slight amount of flimsy clot formed. This is in contrast to the solid clots found within the heart.

*Dog 29-279, Closed Fistula 1 Year, Bleeding Fatal.*—A closed sterile bile fistula was done on Oct. 14, 1930, and the diet thereafter was 300 gm. salmon bread, 100 gm. salmon, 40 gm. Klim. During the following periods the dog was given 100 cc. of dog bile daily by stomach tube: Jan. 28–Feb. 3, 1931; May 12–15; May 26–29; July 6–9; Aug. 6–16; Aug. 23–27. Aug. 6 following a vena puncture a hematoma developed in the neck, and it was for this reason that so much bile was given. No further bleeding occurred until Oct. 10 when again the neck became swollen and the dog had some difficulty in breathing. The skin of the neck was incised and only edematous tissues were found. Oozing from cut vessels continued and whole blood (100 cc.) was transfused and by the end of the procedure bleeding ceased. The dog died during the night.

*Autopsy* (Oct. 11, 1931) discloses extensive hemorrhage deep in the tissues of the neck and infiltrating diffusely through mediastinal tissues and the pericardial sac. There is a small hematoma in the left leg near a vena puncture wound and some hemorrhage in the muscles of the right leg. In the descending and sigmoid colon there are small areas of hemorrhage in the mucosa, and there is a small duodenal ulcer. The bones show no gross evidence of decalcification, and the viscera are normal.

This history is of interest because of the fact that this dog did not exhibit purpuric tendencies until 10 months after operation and there was no serious hemorrhage until a year after operation. It is probable that the bile that was given during the periods indicated prevented bleeding at an earlier date, but that not sufficient bile was given to protect the dog indefinitely.

Dog 26-19, a renal fistula, after 4 months of bile deprivation developed bleeding following vena punctures and was killed. Still another renal fistula dog, 30-266, after being deprived of bile 4½ months died as the result of hemorrhage into tracheal tissues following lodging of a bone.

We have had the opportunity to study some renal fistula dogs which

had been made anemic by various means and in several instances these dogs have developed this purpuric tendency although receiving some bile by mouth. Such anemic dogs require more bile by mouth to prevent the bleeding than do the non anemic fistula dogs.

*Dog 29-66, Anemia, Renal Fistula, Bleeding.*—This dog was studied in the anemia colony with sustained anemia from Aug., 1930, until Feb., 1933, at which time it was allowed to build up its circulating hemoglobin in preparation for operation. Apr. 18, 1933, a gall bladder renal fistula was made, and 6 days later 50 cc. of dog bile was added to the diet of 400 gm. salmon bread, 125 gm. salmon, 50 gm. Klim. The dog was made anemic a month after operation by means of withdrawal of blood from jugular vein, and was kept in a state of continuous anemia. It received daily 50 cc. of dog bile until Dec. 21 and then for a period of 3 months the amount was reduced to 40 cc. daily, and then for a 2 month period it received from 60–70 cc. On May 24, 1934, almost 13 months after operation, it commenced to bleed following vena puncture in the morning and oozed 1–2 ounces during the night but no hematoma formed in the neck. On May 25 fresh normal dog plasma (45 cc.) was given intraperitoneally and for 3 days an extra 100 cc. of dog bile was given by stomach tube. No further bleeding occurred but study of clotting of recalcified plasma on May 28 revealed that it took 11–13 minutes for a solid clot to form in contrast to 2–4 minutes for normal dog plasma. 1 gm. sodium taurocholate as well as 100 cc. of dog bile were added to its ration for the next 11 days.

Fibrinogen determinations on May 26 revealed normal figures. May 29 recalcified plasma clotted in 5–6 minutes. Since this time the dog has continued to get 1 gm. sodium taurocholate plus 50 cc. dog bile with additional 10–20 cc. of ox bile and no further bleeding has occurred although innumerable vena punctures have been done. 5 months later recalcified blood plasma clotted in a solid jelly in 2 minutes, pointing to the beneficial effects of adequate bile. Dog is living and normal at present time.

*Dog 26-18, Renal Fistula, Anemia Colony, Fatal Bleeding.*—A renal fistula operation was performed under ether anesthesia on Oct. 7, 1933. On Oct. 11, dog bile (15 cc.) was added to the diet and 4 days later the amount was increased to 30 cc. daily. From Oct. 25 until Mar. 27, 1934, dog bile (50 cc.) was added daily to the diet of salmon bread, salmon and Klim. Commencing Nov. 7 the dog was made anemic by withdrawal of blood and from Dec. 5 on its hemoglobin hovered around 50 per cent. Numerous jugular vein punctures were made in the routine course of bleedings and on Mar. 27, following a vein puncture oozing began and continued during the day. Whole blood (50 cc.) was transfused late in the afternoon and external bleeding ceased. Next morning (Mar. 28) however there was a large hematoma in the neck, the dog was very weak with labored breathing and the red cell hematocrit was down to 16 per cent. On March 28 it

was found that whole blood would clot but very slowly. Recalcified plasma took 11 minutes to form a jelly in contrast to the very solid clot which formed in 4 minutes in normal plasma. Whole blood (200 cc.) was given intraperitoneally and 65 cc. by vein. The dog died during the night. The finding of interest in the autopsy was a hematoma, 6 x 5 cm., in the neck in close relationship to the jugular vein.

A splenectomized *renal fistula* dog, 30-62, bled intraperitoneally from an abdominal sinus tract 4½ months after being partially deprived of bile. At this time there was severe anemia as the result of *Bartonella* in the blood and this hemorrhage caused death. Another splenectomized *renal fistula* dog, 29-353, at the height of severe anemia, due to *Bartonella* infection, bled from a vena puncture wound 11½ months after operation during which period it had received 50 cc. of dog bile daily. This dog died also as the result of the hemorrhage. Details of autopsies have been described in a previous (11) paper.

Several sterile closed fistula dogs have exhibited this bleeding tendency which develops after 4-5 months of total bile deprivation.

*Osteoporosis with Multiple Fractures.*—Several typical histories of experiments are given in detail and there is a conspicuous uniformity in the story. Obviously the lack of whole bile or some of its constituents is an essential factor in the development of this picture. Since liver feeding is beneficial it is apparent that there is some substance, as yet undetermined, which exerts a protective influence.

Dog 31-303. A gall bladder *renal fistula* was made June 3, 1932, with an uneventful recovery except that the dog did not eat the diet of the kitchen scraps very well so that there was weight loss from 16.6 to 14.2 kilos during the following month and a half. On June 11, 1932, 50 cc. of ox bile was given by stomach tube and continued daily until Dec. 27, except during the month of August when it was given every other day. From July 23 to Aug. 27, the diet consisted of 300 gm. salmon bread, 75 gm. salmon, 30 gm. Klim mixed into a mash with water. At the end of this period the weight was 15.9 kilos or almost the original level. On Dec. 27, the ox bile was discontinued and the diet was changed to one consisting of 300 gm. white bread, 50 gm. Klim and water, and this was consumed completely until Feb. 12. From Feb. 12 to Mar. 4, 1933, the dog tended to leave some food and the weight dropped to 14.1 kilos as a result. A red cell hematocrit of Mar. 7 read 52 per cent and the plasma was jaundiced. The dog appeared healthy and active, but the jaundice had to be accounted for as it is unusual in this type of fistula dog. On Mar. 9, it was noted that the dog urinated frequently but only succeeded in passing a few drops of urine each time. A soft catheter passed into the urethra met with an obstruction which could not be dislodged or passed. By instrumentation several calculi were removed. These calculi were yellow and contained cholesterol by chemical tests. More fragments were passed during the night and the flow of urine increased to normal and the

color was very dark due to the bile. After this episode the dog was given a diet of mixed kitchen scraps which was eaten only moderately well for the next month, but weight was maintained around 14 kilos. From Apr. 5, 1933, to Jan. 19, 1934, it ate all food but from then until death, Feb. 24, it left varying amounts with resulting loss in weight down to 11.4 kilos. On Feb. 1, 1934, the red cell hematocrit was 46.4 per cent with bile staining of plasma indicating mild obstruction to bile flow. All hematocrits have demonstrated maintenance of normal red cell levels during the period of bile deprivation. During the last 3 weeks of life the dog was inactive but made no complaints when being handled until the last day when it whined as pressure was applied lightly to the hind quarters. The thoracic cage at this time was easily compressible and on Feb. 24, 1934, when the dog was placed on its back immediate difficulty in breathing developed and pulsations of heart were very obvious as the sternum tended to collapse inward. When put on the floor again labored breathing disappeared and the dog walked about the room with no distress. Since bone decalcification was now so conspicuous, the dog was killed with gas anesthesia and autopsy performed immediately.

*Autopsy.*—*Dog 31-303, Osteoporosis.*—Serous cavities, heart and lungs are essentially normal, both grossly and histologically. Spleen is small, pinkish-gray with a rather fibrous appearing cut surface. Histologically phagocytic cells containing iron pigment are quite numerous. The mucosa of the intestine is normal throughout, but the muscle coats of the small intestine are colored a buff brown (9). Histologically, there are no changes of importance.

The liver presents normal lobulation but there is slight bile staining. One of the hepatic ducts contains a small pigment calculus. The common bile duct has been ligated and severed and no continuity with the duodenum is demonstrable. The ducts are slightly dilated. The gall bladder is small and the fundus is well healed into the pelvis of the right kidney. The mucosa is studded with minute gray projections. Histologically, the liver architecture is normal, but the bile canaliculi in the liver cells of the central portion of the lobules are plugged with brown colloid material. Liver cells contain lipochrome pigment. The gall bladder has collections of lymphocytes in the submucosa and there are phagocytic cells containing light brown amorphous pigment material. The pancreas grossly is normal except for pigmentation of the character noted in the muscle coats of the intestine.

The right kidney is small and scarred as the result of operation. The pelvis is dilated and there is a calculus composed of pigment and cholesterol which must have caused some obstruction to the free flow of bile. The right kidney is large but normal grossly and histologically. Microscopic sections show the right kidney to be scarred with atrophic or hyalinized glomeruli, and dilated tubules filled with casts. Round cells are scattered in clusters. One region shows more normal kidney tissue. A portion of gall bladder healed into the kidney pelvis is present. The ureters and urinary bladder are normal grossly, and histologically only a few round cells are found in the submucosa of the bladder. The prostate is normal in gross but histologically there are a few collections of lymphocytes between the acini.

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The skeletal system is very interesting due to the decalcification. The ribs are easily compressible, bend but do not break, and near the costal junction each rib from the 3rd to the 8th on the right side shows a greenstick fracture and on the left side each rib from the 3rd to the 9th. The vertebral bodies and scapulae cut easily with scissors, and the cortices of the long bones are thinned and split easily.

Histologically, the cortex of the femur shows very large Haversian spaces some of which contain fibrous tissue. The bone adjacent to the canals shows evidence of decalcification. The vertebral bones show delicate trabeculae with some osteoclastic activity. The ribs show more striking changes. The cortical bone is thin and its continuity broken with replacement of bone by connective tissue. In the region of the fractures all normal architecture is disrupted, and one finds callus, new bone, bone trabeculae being eroded by osteoclasts and fibrosis of marrow spaces. Marrow is hyperplastic but still contains some fat.

*Dog 29-329, Renal Fistula plus Splenectomy, Anemia, Ox Bile Feeding, Osteoporosis.*—The dog was operated upon under ether anesthesia Sept. 29, 1933, and a gall bladder renal fistula made plus splenectomy. From Oct. 2 to Nov. 10, 1933, the dog was given 50 cc. of dog bile by stomach tube and then changed to 50 cc. of ox bile which it continued to receive 6 days a week until Nov. 28, 1934. The diet from Oct. 9 to Nov. 13, 1933, was 250 gm. salmon bread, 75 gm. salmon and 30 gm. Klim, and from Nov. 13, 1933, to Nov. 22, 1934, the dog was fed 700 gm. of kennel diet. During the period the dog was in the anemia colony it received salmon bread diet.

This dog for months exhibited periods of hemoglobin destruction and excess bile pigment production related to infection with *Bartonella* (8). On June 27, 1934, the dog was given 210 mg. of neosalvarsan intravenously with sterilization of the blood stream of the *Bartonella* and thereafter it had a normal hemoglobin level and bile pigment output. From Nov. 22, 1934, until Jan. 29, 1935, the dog was studied in the anemia colony and by bleedings from the jugular vein its hemoglobin had been lowered from 106 per cent to 39 per cent. On Dec. 1, the ox bile (50 cc.) was replaced by 1 gm. of sodium taurocholate fed with the food and it received this amount daily until Dec. 18 when it was given 2 gm. daily. On Dec. 18 recalcified plasma clotted in 3 minutes and on Dec. 26 in 7 minutes. From Dec. 26 until Jan. 8 ox bile (40 cc.) was given with the food. Sodium taurocholate (2 gm.) and ox bile (40 cc.) were fed from Jan. 8 to Jan. 22 when the ox bile was discontinued. On this date the plasma was clear and when recalcified it clotted in 8½ minutes. From Jan. 22 to 29 the dog ate poorly and on the morning of the latter date was found to be very weak. The dog had been anemic since Dec. 31 with the hemoglobin about 50 per cent. Hidden bleeding causing too severe an anemia was suspected and the dog was transfused at once with whole blood (60 cc.) but death followed shortly thereafter. The plasma however was definitely jaundiced. All during this 14 months as a renal fistula the dog's weight had been well maintained and there had never been any spontaneous bleeding.

*Autopsy.*—The serous cavities, heart and lungs are normal both grossly and histologically. The heart is full of firmly clotted blood. The liver is dark reddish brown in color with normal lobulation. The common bile duct is ligated and completely severed from the duodenum. The fundus of the gall bladder is firmly healed into the pelvis of the right kidney. The ducts and gall bladder are not dilated. Histologically the liver shows some phagocytic cells filled with dark brown pigment in the portal regions and occasionally elsewhere in the lobule. Some bile canaliculi contain brown colloid material. The gall bladder appears normal.

The stomach is normal but in the duodenum there is a small ulcer 3–4 mm. in size. The mucosa of the remainder of the intestine is normal, but the muscle coats of the small intestine are colored a buff brown. Histologically there is a small ulcer of the duodenum with a few mononuclear cells in the submucosa which however shows no destruction. The pancreas is normal except for the pigmentation similar to that in the intestinal coats.

The right kidney is scarred at the operative site but otherwise is normal. The left kidney is normal except for slight hypertrophy. Histologically the right kidney shows round cells in the submucosa of the pelvis and some collections in the parenchyma in the scarred area. The bladder and prostate are normal except histologically there are round cells in the stroma of the prostate. Lymph glands at the hilum of the liver are large, brownish in color, and histologically there are phagocytes containing iron pigment.

The bony skeleton shows decalcification. The ribs are brittle and on the left side the 4th, 5th and 6th ribs each show two small bulges which appear to be healed fractures, and in the 9th there is one such fracture. On the right side the 4th, 5th and 6th ribs show two fractures and the 2nd, 3rd, 7th and 8th one each. The vertebral bodies can be cut easily with the knife, and the long bones are brittle and easily split. The marrow shows hyperplasia due to the secondary anemia. Histologically there is definite thinning of the cortex and trabeculae of the ribs. Lines of fracture are apparent and in these areas there is much cartilage, new bone formation and fibrosis of the marrow spaces. Numerous large osteoclasts are to be seen. Vertebral bone trabeculae are delicate and show thinning out but marked decalcification is not evident. Marrow is hyperplastic as the result of the experimentally induced anemia.

This dog is of particular interest since it had received a kennel diet ration with 50 cc. of ox bile for most of its existence as a fistula animal, and still there was evidence that decalcification had progressed. The osteoporosis was not as severe however and healing of fractures occurred. The ox bile had apparently protected this dog from purpura and bleeding.

*Dog 31-27, Biliary Obstruction, Osteoporosis, No Bile Given, Metastatic Calcification.*—The dog was operated upon June 1, 1932, and a closed sterile bile fistula made plus splenectomy. Recovery was uneventful and bile drained freely. A month later on 3 successive days, the dog chewed the rubber bag into which the bile



drains, so the rubber tubing was cut off close to the abdomen and the dog set aside for bile deprivation studies. Jaundice developed as the fistula tract promptly became obstructed. No bile was given by mouth. A diet of mixed kitchen scraps was fed and the dog ate this completely and continued lively until April, 1933, at which time it became listless and just lay quietly in the cage, but continued to eat. The last week of life it refused to stand and when placed on its feet would immediately slump down. The rib cage was easily compressible but at no time did the dog indicate that discomfort was caused by palpation over the ribs or long bones. On May 4, 1933, 11 months after operation, it was killed with gas anesthesia. During this time there had been gradual loss of weight from 13.6 kilos to 6.8 and the hair had become rough. No spontaneous bleeding had ever occurred.

*Autopsy* was performed at once and positive findings are noted. There is evidence of marked loss of weight, and the tissues throughout are bile-stained. The bile fistula opening in the side is healed and there is complete obstruction of the common duct. The serous surface of the peritoneal cavity is smooth but there are fibrous adhesions about the liver and bile duct and a fibrous sheath which had formed about the rubber fistula tubing extends from the end of the common bile duct to the lateral abdominal wall.

The heart is of normal size and external appearance. The endocardium of the right auricle and ventricle is smooth and the tricuspid and pulmonic valves are delicate. In the first portion of the pulmonic artery there are small rough vegetative-like calcified nodules, 1 to 2 mm. in size, involving the intimal surface. In the left auricle just above the valve more of these calcified nodules are present and a few are present on the mitral leaflets. The endocardium of the left ventricle and the aortic cusps are normal. The myocardium appears normal. Throughout the length of the aorta there are scattered small calcified nodules, again apparently involving only the intima. Histologically, the intimal layer of the pulmonary artery is thickened by connective tissue and there are masses and streaks of calcium deposit but the media is not involved. The sections of aorta present a similar picture. The endocardium of the left auricle is slightly thickened and calcified areas involving some of the elastic fibers are found. The myocardium is normal. The blood clots quickly and firmly indicating no abnormality in clotting. The lungs show partial atelectasis of the lower lobes, and histologically the alveolar walls contain calcium in abundance. There are scattered phagocytic cells filled with golden yellow pigment. The liver is deep green in color with lobulation distinct beneath the capsule. On section the periportal connective tissue is conspicuous and near the hilum about the larger bile ducts distinctly increased. The hepatic and common bile ducts are distended with grayish-white, thick material. The common bile duct is ligated and no communication with the duodenum is found. Histologically there is some increase in periportal connective tissue and bile duct proliferation is apparent with accumulations of round cells about them. Bile canaliculi in centrally located liver cells are plugged with brown colloid material. In some portal areas, there are large

phagocytic cells, filled with granules of brown pigment, which gives a positive stain for iron. The common bile duct has a thick fibrous wall with round cells and phagocytic cells containing golden yellow pigment and the mucosa has collections of round cells. Arteries show calcium deposits in the intima with some replacement of the media.

The pancreas is normal grossly and histologically but there are arteries which show calcification of portions of the wall. The gastro-intestinal tract presents a normal appearance grossly and histologically except for calcification of the intima and media in some of the arteries.

The kidneys grossly are normal but sections show calcification of the intimal lining of arteries without any thickening of this layer. Some epithelium of the collecting tubules has undergone calcification. Bile-stained casts are present in some tubules. The urinary bladder mucosa contains numerous large blebs due to cystitis emphysematosa, and histologically the mucosa shows polymorphs lying in the submucosa between these large blebs.

Lymph glands at the hilum of the liver are enlarged, measuring 3 cm. in length and have a reddish-brown color. Histologically, the sinuses are large and contain many phagocytic cells filled with light yellow pigment and some granules of darker brown pigment which gives a stain for iron.

The bony skeleton presents most interesting changes. The rib cage is deformed due to softening of the ribs with multiple fractures. Every rib shows fractures, some 2 or 3 and others as many as 5 or 6. The fractures present themselves as white knobs and are more of a greenstick type rather than complete breaks. The ribs are very elastic and bend easily but do not break. The vertebral bodies are very soft and easily cut with the knife. The cortex of the long bones is thinned to about 2 mm. in thickness, and the bones can be split by applying some pressure to the knife. Marrow is red throughout the bones. Scapulae and pelvic bones are very delicate and easily cut. Histologically, the cortex of the ribs is thinned and in places bone is replaced by connective tissue. Lines of fracture are apparent and in this region the normal architecture is gone and there is osteoid tissue, bone fragments and fibrosis of marrow spaces. The vertebral sections show very delicate bone trabeculae with erosion crypts apparent. The cancellous portion of the long bones shows a thin cortex with delicate bone trabeculae. Large osteoclasts are not conspicuous. The marrow is very hyperplastic and is similar to that seen in prolonged secondary anemia except that the neutrophilic series is most conspicuous with adult polymorphonuclears numerous. Megakaryocytes are plentiful.

This *metastatic calcification* of vessels and stroma of organs is probably the result of the mobilization of calcium from the bones due to the lack of absorption of vitamin D from the intestine. Wells (16) in 1915 reviewed the literature and reported a somewhat similar picture in a case of human myelogenous leucemia where there was bone

drains, so the rubber tube for bile deprivation study became obstructed. No scraps was fed and the dog died in 1933, at which time it continued to eat. The last 4 feet would immediately stop at no time did the dog hit the ribs or long bones. Operated with gas anesthesia. Duration 13.6 kilos to 6.8 and the bile never occurred.

*Autopsy* was performed with evidence of marked loss of weight. The bile fistula opening in the common duct. The stomach there are fibrous adhesions and had formed about the rubber bile duct to the lateral abdominal wall.

The heart is of normal size, right auricle and ventricle is delicate. In the first portion vegetative-like calcified nodules face. In the left auricle just are present and a few are in the left ventricle and the aorta normal. Throughout the lungs nodules, again apparently in layer of the pulmonary artery masses and streaks of calcium of aorta present a similar picture thickened and calcified areas myocardium is normal. The normality in clotting. The histologically the alveolar wall is filled with phagocytic cells filled with in color with lobulation distinct connective tissue is conspicuous distinctly increased. The lungs grayish-white, thick material communication with the duodenum periportal connective tissue contains clusters of round cells about the are plugged with brown color

it was given daily in amounts indicated: Mar. 28-30, dog bile 50 cc.; Feb. 23-27, 1933, dog bile 60 cc.; Apr. 29-May 7, dog bile 50 cc.; June 11-24, ox bile 100 cc. From June 2-15, pig liver 300 gm. was added to the diet and 100 gm. pig liver from Apr. 29-May 7. The fistula obstructed June 12, 1933. There had never been any anemia or spontaneous bleeding, and the weight after 1½ years' existence as a fistula dog was 15.7 kilos as contrasted with 15 kilos the day of operation. 3 months after obstruction the dog had several severe gastro-intestinal hemorrhages which caused death. The anatomical diagnosis was: obstructed bile fistula; generalized jaundice, acute cholangitis; two bleeding ulcers in duodenum; infected infarcts of spleen and kidneys; pigmentation of small intestine and pancreas. The blood clotted immediately and the bones were firm in consistency.

#### DISCUSSION

Spontaneous bleeding with purpura is of particular interest and many attempts to control this tendency have been made as these dogs developed the bleeding tendency. Under the conditions of these experiments it is clear that there is a lack of something needed for normal coagulation. As this lack of something develops we note first a delayed blood clotting time which eventually leads to spontaneous bleeding from mucous membranes and trivial surface injuries. This condition can be relieved temporarily by whole blood transfusion. Whole bile feeding in considerable amounts will check the bleeding and remedy the lack of something, and the cure is permanent if the bile feeding is continued in adequate amounts daily. Dog bile is more effective in these experiments than ox bile. Bile salt by itself does not appear to be effective. We hope to determine what constituents of whole bile are responsible for this condition. When common bile duct obstruction is present with inevitable jaundice, this spontaneous bleeding does not develop indicating that abnormal retention of bile within the body is effective to prevent the development of this condition. From analysis of blood coagulation factors it seems highly probable that prothrombin is lacking or greatly diminished in the circulating blood when spontaneous bleeding develops. Experiments dealing with this point will be published in the near future. This knowledge is of great value in handling the bile fistula dog to prevent or cure serious complications and it may also be of service eventually in the control or cure of certain types of human diseased states associated with bleeding.

destruction. This condition although not common is found where there has been extensive destruction of bone as occurs in tuberculosis or neoplasms of bones and leucemia.

### *Control Observations*

In contrast to the above animals, we have had gall bladder renal fistula dogs remain in health indefinitely upon a diet of salmon bread plus 50 cc. of dog bile by stomach tube.

Dog 29-331 was operated upon under ether on July 25, 1930, and a gall bladder renal fistula made. At that time the dog weighed 14.6 kilos while just before death in March, 1934, it weighed 16 kilos. Aug. 15, 1930, there was a hemoglobin of 103 per cent contrasted with 118 per cent on Mar. 8, 1934, and at no time had there ever been an anemia. The basal diet was 400 gm. salmon bread, 75 gm. salmon and 30 gm. Klim. During nine periods varying from 3 to 4 weeks 200-300 gm. of liver had been added to the diet. Dog bile 50 cc. was given daily by stomach tube. Bleeding from the mucous membranes or from vena puncture wounds had not occurred. The dog was approaching a senile state and was killed by gas anesthesia.

*Autopsy* shows essentially normal organs with the fistula tract patent. The common bile duct is completely severed and no new tract had formed between the duct and the duodenum. The bones are solid and show no evidence of decalcification. The muscle coats of the small intestine, the mesenteric lymph glands and the pancreas are pigmented a buff brown.

Another dog, 31-267, has carried a renal fistula since Mar. 31, 1932, and at the present time—May, 1935—is in excellent clinical condition. The weight has been constant between 13.1 and 12 kilos. The diet was 250 gm. salmon bread, 75 gm. salmon, 30 gm. Klim and dog bile (50 cc.) was given by stomach tube until July, 1934, at which time ox bile 50 cc. was substituted. The bile has been given 6 days a week. Although it has been made anemic over long periods of time by means of acetyl phenylhydrazine, the hemoglobin has promptly returned to normal levels on cessation of the drug. There has never been any spontaneous bleeding and Feb. 21 recalcified plasma clotted in  $6\frac{1}{2}$  minutes.

Although the usefulness of closed sterile bile fistula dogs generally is of about 5 months' duration, we have had two dogs that were used in bile pigment and bile salt metabolism studies for 1 year and  $1\frac{1}{2}$  years respectively, Dogs 31-203 and 29-279.

*Dog 31-203, Closed Fistula  $1\frac{1}{2}$  Years, Obstruction, Duodenal Ulcers.*—A closed sterile bile fistula was made on Jan. 26, 1932, and the dog thereafter was fed a basal diet of 300 gm. salmon bread, 75 gm. salmon and 30 gm. Klim. All bile was excluded from the intestinal tract except during the following periods when

it was given daily in amounts indicated: Mar. 28-30, dog bile 50 cc.; Feb. 23-27, 1933, dog bile 60 cc.; Apr. 29-May 7, dog bile 50 cc.; June 11-24, ox bile 100 cc. From June 2-15, pig liver 300 gm. was added to the diet and 100 gm. pig liver from Apr. 29-May 7. The fistula obstructed June 12, 1933. There had never been any anemia or spontaneous bleeding, and the weight after 1½ years' existence as a fistula dog was 15.7 kilos as contrasted with 15 kilos the day of operation. 3 months after obstruction the dog had several severe gastro-intestinal hemorrhages which caused death. The anatomical diagnosis was: obstructed bile fistula; generalized jaundice, acute cholangitis; two bleeding ulcers in duodenum; infected infarcts of spleen and kidneys; pigmentation of small intestine and pancreas. The blood clotted immediately and the bones were firm in consistency.

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The healthy renal bile fistula can be maintained in perfect condition and weight equilibrium on a salmon bread diet plus 50 cc. of whole dog bile daily by mouth. If this same dog is made anemic by bleeding and kept in an anemic state by blood withdrawal we must increase the intake of dog bile or the fistula dog will develop spontaneous bleeding. A dose of 75 cc. whole bile daily may now be required to preserve a normal condition. It would appear probable that the circulating plasma contains some of the unknown substance which accumulates as a result of the bile feeding and therefore removal of large amounts of plasma so depletes this supply that a larger intake of bile is needed.

Osteoporosis or decalcification of the skeleton is also of especial interest and obviously has a very important relationship to bile salt metabolism. As the bones are decalcified there may appear abnormal calcium deposits in the vessels and stroma of lungs and viscera. This was noted many years ago by Wells (16).

Schmidt (3) and others have noted that the bile salts favored the absorption of vitamin D and gave an adequate explanation of the osteoporosis and its cure by bile salt feeding.

The observation that liver feeding would prevent the development of osteoporosis in open bile fistula dogs was made repeatedly in the Hooper Foundation and reported (19, 17) but not studied in detail. It is not possible to give a correct explanation of this observation but it may be proper to speculate as to the mechanism of this reaction. It may be argued that the liver contains an unknown substance or a compound of vitamin D either of which is absorbable through the intestine and prevents the decalcification. It would be of some interest to test fractions or extracts of whole liver to ascertain whether a potent fraction could be found which is capable of preventing bone decalcification under such conditions.

#### SUMMARY

A clearer understanding of the various abnormalities which may develop in relation to the experimental or clinical bile fistula will be of value to the laboratory worker as well as to the physician and surgeon.

A better comprehension of these diseased conditions will make for

a saner analysis of the great mass of experimental data relating to the various types of bile fistula. Too frequently in the literature the bile fistula material is used to debate a physiological state whereas in reality the animal is in a pathological condition.

It is possible but not easy to keep a bile fistula dog in a normal state for months or years if proper attention is given to the diet and physical state.

The most significant abnormalities are—intestinal disturbances, spontaneous bleeding, osteoporosis, cholelithiasis and duodenal ulcer. Three types of bile fistula were used in our experiments and each one has its advantages and disadvantages.

Intestinal intoxication is best controlled by diet, by whole bile or bile salts or combinations of dog and ox bile. Spontaneous bleeding seems to be due to the loss of something by way of the bile and this can be prevented by bile feeding. The blood deficiency appears to be a lack of prothrombin.

Osteoporosis appears inevitably after many months if bile is excluded from the intestine. This state is related to the lack of absorption of vitamin D. It is of some interest that liver feeding will prevent it.

Duodenal ulcers and cholelithiasis are common in bile fistula dogs and absolute control or prevention is not easy.

It may be restated that bile secretion into the intestine is necessary for normal health and even for actual continuation of life beyond a few months' period. Some of these experimental data should be of value to physicians and surgeons in the care and study of human fistula cases and should emphasize the necessity of prompt dietary control.

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# THE PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES: ELICITATION OF LOCAL REACTIVITY BY WAY OF THE VASCULAR SYSTEM

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PLATES 26 AND 27

(Received for publication, July 29, 1935)

In the phenomenon of local skin reactivity to bacterial filtrates the state of reactivity is obtained by means of an intradermal injection of a potent bacterial filtrate. It seemed of interest to determine whether local reactivity could be elicited by way of the vascular system. This report deals with experiments on rabbit ears in which the question was investigated.

## *Methods*

Twenty-four to 48 hours prior to the experiments the skin of the rabbit's ear was epilated by means of barium sulfide. When deemed necessary, the circulation was stopped by applying a clamp to the base of the ear for periods of time indicated in the respective protocols. The efficiency of the clamp was checked by injection of a dye of slow diffusibility recently used extensively by Rous and his coworkers (1), namely Niagara sky blue, which was prepared according to the method described by Rous (1).

*Rabbit 5-38.*—1 cc. of a 17 per cent solution of the dye was injected into the marginal vein of the left ear. A few seconds later, left and right ears and the conjunctiva of the eyes stained blue. A little later the entire body became blue, showing, however, during the following hour a more intense color in both ears and eyes than in the rest of the body.

*Rabbit 5-39.*—A clamp was applied to the base of the left ear. 5 minutes later, 1 cc. of 17 per cent solution of Niagara sky blue was injected into the marginal vein of the same ear. The left ear became blue a few seconds later but the rest of the body did not stain. The clamp was removed 13 minutes after the injection of the dye. At this time, the color spread instantaneously to the right ear and then to the conjunctiva of the eyes. Approximately 2 minutes after the removal of the clamp, the body began to stain blue. For the following period of 1 hour

the staining of the left ear remained more intense than of the right ear and eyes.

As is seen from these experiments, the application of the clamp used was efficient in stopping entirely the spread of the substance injected from the clamped ear into the general circulation.

### *Phenomenon of Local Skin Reactivity to Bacterial Filtrates in Rabbit's Ear*

Attempts to reproduce the phenomenon of local skin reactivity to bacterial filtrates in the rabbit's ear have been made previously by Gratia and Linz (2), Klein in these laboratories (3), and recently by Alechinsky (4).

When appropriate doses of the toxic material are used for the preparatory and provocative injections, strong reactions can be obtained in the rabbit's ear. The reactions may be well circumscribed and intensely hemorrhagic. Very frequently, however, there is a tendency towards generalization of the reaction through the entire ear and sometimes, as also reported by Gratia and Linz (2), there may be an extension of the reaction to the opposite ear. Frequently there is observed blue discoloration through the entire ear. Fig. 1 illustrates the typical reaction of the ear. Fig. 2 presents the histological appearance of the tissues at the prepared site. As is seen, the reaction is accompanied by hemorrhage, pronounced capillary thrombosis, subsequent severe necrosis, and inflammation. Fig. 3 demonstrates the microscopic appearance of discolored parts of the ear well removed from the local reaction. The conspicuous feature of the discolored tissues is extensive thrombosis in the venules.

Before attempting the series of studies planned in this investigation, it seemed important to compare the susceptibility of the rabbit's ear to the phenomenon with that of the abdominal skin on which all the previous studies were carried out.

*Comparative Titrations.*—All titrations described were done within 1 month, using the same preparation; i.e., *B. typhosus* "agar washings" filtrate (T.1986). The preparatory intradermal injections consisted each of 0.25 cc. of undiluted filtrate. The doses used in the intravenous injections ranged from 5 to 80 reacting units. Each intravenous dose was tested in a group of three rabbits. The results are summarized in Table I.

As is seen from Table I, severe reactions were consistently obtained in the skin of the abdominal wall beginning with the intravenous

dose of 2 reacting units. The incidence of reactions was the same in groups receiving the provocative injection into the right and into the left marginal ear veins.

When the intradermal injections were given into the skin of the ear and the provocative injection into the marginal vein of the same ear, at least 20 reacting units were necessary for the elicitation of the

TABLE I

*Comparative Titrations in the Skin of the Ear and of the Abdominal Wall*

| Group No. | Prepared skin site              | Toxin and dose used for preparatory injection* | Vein used for provocative injection | Dose used for provocative injection | Reactions following provocative injection |
|-----------|---------------------------------|--|-------------------------------------|-------------------------------------|---|
| 1         | Upper right quadrant of abdomen | 0.25 cc. B. TyT <sub>L</sub> † T. 1986         | Right marginal ear vein             | 2 units                             | 1/2†                                      |
| 2         | " "                             | 0.25 " " "                                     | " "                                 | 5 "                                 | 2/1                                       |
| 3         | " "                             | 0.25 " " "                                     | " "                                 | 20 "                                | 2/0                                       |
| 4         | " "                             | 0.25 " " "                                     | Left marginal ear vein              | 2 "                                 | 1±/2                                      |
| 5         | " "                             | 0.25 " " "                                     | " "                                 | 5 "                                 | 1/2                                       |
| 6         | " "                             | 0.25 " " "                                     | " "                                 | 20 "                                | 2, 1±/0                                   |
| 7         | Left ear                        | 0.25 " " "                                     | " "                                 | 5 "                                 | 0/2                                       |
| 8         | " "                             | 0.25 " " "                                     | " "                                 | 20 "                                | 2±/1                                      |
| 9         | " "                             | 0.25 " " "                                     | " "                                 | 40 "                                | 1/2                                       |
| 10        | " "                             | 0.25 " " "                                     | " "                                 | 60 "                                | 2/1—1 died                                |
| 11        | " "                             | 0.25 " " "                                     | Right marginal ear vein             | 5 "                                 | 0/3                                       |
| 12        | " "                             | 0.25 " " "                                     | " "                                 | 20 "                                | 0/3                                       |
| 13        | " "                             | 0.25 " " "                                     | " "                                 | 40 "                                | 0/3                                       |
| 14        | " "                             | 0.25 " " "                                     | " "                                 | 60 "                                | 2/1                                       |

\* 0.25 cc. was injected intradermally.

† Abbreviation B. TyT<sub>L</sub> designates "agar washings" filtrates of *B. typhosus*, Strain T<sub>L</sub> cultures.

‡ The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum of both indicates the total number of rabbits in each group.

reactions. Rabbits with prepared ears receiving provocative injections into the marginal veins of the non-prepared ears, gave reactions only when at least 60 reacting units were used. As is seen from these titrations, the skin of the ear is considerably more resistant to the phenomenon than the skin of the abdomen. It is of interest that ten times the provocative dose is required if it is given into the vein of the prepared ear and thirty times the dose if it is given into

the vein of the non-prepared ear. The obvious interpretation of this observation is that a certain amount of toxin injected into the vein of the non-prepared ear is lost in the general circulation before reaching the skin of the prepared ear.

*Elicitation of Local Reactivity in the Rabbit's Ear by Way of the Vascular System*

Various attempts were made to prepare the tissue of the ear by an intravenous injection instead of intradermal. In planning these experiments the above information was taken into consideration.

*Group 1.*—1 cc. of meningococcus Group III (44B.) "agar washings" filtrate (T.1968) diluted 1:50 (80 reacting doses) was injected into the marginal vein of the left ear of each of three rabbits tested. 24 hours later, the filtrate was injected into the right marginal ear vein in a dose of 50 reacting units, per kilo of body weight. No reactions were observed.

*Group 2.*—The ears of three rabbits were clamped off at the base. 1 cc. containing 50 reacting units of meningococcus Group III (44B.) "agar washings" filtrate (T.1968) was injected into the left marginal ear vein of each rabbit. The clamps were removed 5 minutes after the intravenous injections were completed. 24 hours later, 50 reacting units (per kilo of body weight) of the same filtrate were injected into the left marginal vein. No reactions followed.

*Group 3.*—Clamps were applied to the base of the left ear of three rabbits. 1 cc. containing 100 reacting units of *B. typhosus* "agar washings" filtrate (T.1968) was injected into the left marginal vein of each rabbit. Clamps were removed 5 minutes after the intravenous injection. 24 hours later, 100 reacting units (per kilo of body weight) were injected into the left marginal ear veins. No reactions followed.

As is seen from these experiments, preparatory intravenous injections of potent filtrates fail to elicit the state of reactivity of the phenomenon under discussion. The same doses given intradermally elicited this state in most rabbits tested. Unfortunately, larger doses could not be used because of the lethal effect of these preparations. It was assumed, then, that the perivascular preparation was necessary for elicitation of the state of reactivity. In experiments, such as the above, under conditions of normal resistance, the capillaries may not allow the diffusion of the preparatory factors into the tissues when given intravenously. In view of this assumption, it was decided to accompany the preparatory intravenous injections of the toxin by various agents known to modify capillary permeability, as follows:

*Preparatory Intravenous Injections of Toxins Accompanied by  
Application of Heat*

In these experiments sausage shaped rubber bags filled with water of the desired temperature were firmly applied to both sides of the ear for 5 minutes and the preparatory injections were given into the marginal vein. In some experiments the ear was clamped off at the base simultaneously with the application of heat and the clamps were removed 5 minutes after the intravenous injections were completed. The provocative injections were given into the vein of the same or of the opposite ear. The results of these experiments are summarized in Table II.

In the experiments recorded in Table II, clamped and non-clamped ears exposed to 45°, 50°, and 55°C. for 5 minutes and injected with various amounts of active bacterial filtrates intravenously, showed immediate intense hyperemia with subsequent swelling and moderate hyperemia 24 hours after the treatment.

Provocative injections of toxin in the various amounts recorded in Table II, elicited no secondary reactions in non-clamped ears.

The preparatory intravenous injections of potent preparations given into the veins of ears exposed to 45°, 50°, and 55°C. and clamped off at the base were capable of eliciting the state of reactivity of the phenomenon under consideration. Reactions following the provocative injections were intense. Diffuse hemorrhages throughout the entire ear were accompanied frequently by deep cyanosis. Microscopically there was pronounced thrombosis in the venules (Fig. 3). There were also observed petechial hemorrhages in various parts of the ear and sometimes in portions far removed from the site of the injected vein. In the gross, these petechiae closely resembled purpuric spots seen in the skin of human cases of meningococcemia.

As is seen from Table II, ears exposed to 45°C. for 5 minutes gave definite reactions following the provocative injection, provided at least 50 reacting units were used for the preparatory intravenous injection. When exposure to 50°C. was combined with the preparatory injection of toxin, distinct reactions were seen with as little as 5 units, provided the provocative injection was given into the same vein. It is curious that a preparatory dose of 25 reacting units in ears exposed to 50°C. failed to elicit the state of reactivity. This observation is difficult to explain and the experiment should be repeated. Provocative injections of toxins into the ears prepared by larger doses



TABLE II  
*Preparatory Intravenous Injections of Toxins Accompanied by Application of Heat*

| Group No. | Time during which ear was clamped | Dose of preparatory injection of toxin into left marginal ear vein | Application of heat to the left ear | Reactions following preparatory intravenous injection | Vein used for provocative intravenous injection |                         | Dose of provocative injection | Reactions following provocative injection  |
|-----------|-----------------------------------|--|-------------------------------------|---|---|-------------------------|-------------------------------|--|
|           |                                   |  |                                     |   | Right marginal ear vein                         | Left marginal ear vein  |                               |  |
| 1         | None                              | 50 units Mg. 44B.* T. 1968   | Bags 45°C. 5 min.                   | 3†—negative   | Right marginal ear vein                         | Left marginal ear vein  | 25 units per kilo             | 2†—negative; 1—died  |
| 2         | 5 min.                            | 25 "   | " 45°C. 5 "                         | 3 "   | Right marginal ear vein                         | Left marginal ear vein  | " "                           | 3—negative   |
| 3         | 5 "                               | 50 "   | " 45°C. 5 "                         | 3 "   | Right marginal ear vein                         | Right marginal ear vein | " "                           | 2—hemorrhages and thrombosis of marginal veins; 1—negative                                   |
| 4         | None                              | 80 "   | " 50°C. 5 "                         | 3 "   | Left marginal ear vein                          | Left marginal ear vein  | " "                           | 3—negative   |
| 5         | 5 min.                            | 1 "  | " 50°C. 5 "                         | 3—ears swollen and hyperemic; 3—negative              | " "   | " "                     | " "                           | 1—hemorrhage along marginal vein; 5—negative   |
| 6         | 5 "                               | 5 "  | " 50°C. 5 "                         | 6—negative  | Right marginal ear vein                         | Left marginal ear vein  | " "                           | 2—diffuse hemorrhagic reaction over entire right ear; 2—doubtful; 2—negative                 |
| 7         | 5 "                               | 5 "  | " 50°C. 5 "                         | 3—swellings   | Left marginal ear vein                          | Right marginal ear vein | " "                           | 3—negative   |
| 8         | 5 "                               | 25 "   | " 50°C. 5 "                         | 3—negative  | Right marginal ear vein                         | Left marginal ear vein  | " "                           | " ; 1—died   |
| 9         | 5 "                               | 25 "   | " 50°C. 5 "                         | 3—swellings   | Left marginal ear vein                          | Right marginal ear vein | " "                           | 2—diffuse cyanosis; 1—negative   |
| 10        | 5 "                               | 50 "   | " 50°C. 5 "                         | 1—swelling; 2—negative                                | Right marginal ear vein                         | Left marginal ear vein  | " "                           | 2—intense diffuse hemorrhagic reaction over entire ear; 1—negative                           |
| 11        | 5 "                               | 80 "   | Water bath 50°C. 5 min.             | 3—swellings   | Right marginal ear vein                         | Left marginal ear vein  | " "                           | 1—tip cyanosis (6 x 5 cm.) intense and sharply demarcated; 1—small cyanotic area; 1—negative |
| 12        | 5 "                               | 50 "   | Bags 55°C. 5 min.                   | 3 "   | Right marginal ear vein                         | Left marginal ear vein  | " "                           | " "  |

\* Abbreviation Mg. 44B. designates "agar washings" filtrates of meningococcus Group III cultures.

† Number of rabbits.

(i.e., 50 and 80 reacting units in ears exposed to 50° and 55°C.) gave intense and diffuse reactions.

It may be concluded from these experiments that preparatory intravenous injections of toxins are capable of eliciting the state of reactivity in the skin of the ear provided they are combined with temporary stasis (clamping) and thermal hyperemia.

### *Preparatory Intravenous Injections of Toxins Accompanied by Chilling*

*Protocol 1.*—The left ears of three rabbits were chilled by means of ice bags applied to both sides for a period of 10 minutes. About 2 minutes after completion of chilling, when the ears became flushed, they were clamped off at the root and 80 reacting units of meningococcus Group III (44B.) "agar washings" filtrate (T.1968) were injected into the marginal veins. The clamps were kept on for 2 minutes following the intravenous injection. 24 hours later the rabbits were injected into the marginal veins of the same ears with 25 reacting units of the above toxic filtrate. No reactions were observed.

*Protocol 2.*—In this experiment the left ears were clamped off at the base and 80 reacting units of meningococcus, Group III (44B.) "agar washings" filtrate (T.1968) were injected into the left marginal veins. Immediately after completion of the intravenous injection, ice bags were applied to the left ears. 5 minutes later the bags were removed and the clamps released. The ears appeared distinctly hyperemic shortly afterwards. 24 hours later the left marginal veins were injected intravenously with 25 reacting units, per kilo of body weight, of the same toxin. One rabbit died and the two surviving rabbits showed no reactions.

As is seen from these experiments, preparatory intravenous injections were given into the clamped ears in adequate doses. The ears were chilled prior to and following the intravenous injection for periods of 5 and 10 minutes. Distinct hyperemia followed the treatment. The injections failed, however, to elicit the state of reactivity.

### *Preparatory Intravenous Injections of Toxins in Xylol Treated Ears*

*Protocol 1.*—The left ears of six rabbits were clamped off at the base and rubbed with xylol until the veins became very prominent. Immediately afterwards, rabbits received 50 reacting units of meningococcus, Group III (44B.) "agar washings" filtrate (T.1968) into the marginal veins of treated ears. The clamps were removed 5 minutes after the intravenous injections were completed. 24 hours later the ears appeared swollen and one was slightly hemorrhagic along the marginal vein. All the rabbits received 50 reacting units of the same toxin, per kilo of body weight, into the right marginal ear veins 24 hours later. In the rabbit

TABLE II  
Preparatory Intravenous Injections of Toxins Accompanied by Application of Heat

| Group No. | Time during which ear was clamped | Preparatory  |   | Application of heat to the left ear | Reactions following preparatory intravenous injection | Vein used for provocative intravenous injection |                         | Dose of provocative injection | Reactions following provocative injection  |
|-----------|-----------------------------------|--|---|-------------------------------------|---|---|-------------------------|-------------------------------|--|
|           |                                   | Dose of preparatory injection of toxin into left marginal ear vein | Dose of preparatory injection of toxin into right marginal ear vein |                                     |   | Right marginal ear vein                         | Left marginal ear vein  |                               |  |
| 1         | None                              | 50 units Mg. 44B.* T. 1968   | "   | Bags 45°C. 5 min.                   | 3†—negative   | Right marginal ear vein                         | Left marginal ear vein  | 25 units                      | 2†—negative; 1—died  |
| 2         | 5 min.                            | 25 "   | "   | " 45°C. 5 "                         | 3 "   | Left marginal ear vein                          | "                       | "                             | 3—negative   |
| 3         | 5 "                               | 50 "   | "   | " 45°C. 5 "                         | 3 "   | Right marginal ear vein                         | "                       | "                             | 2—hemorrhages and thrombosis of marginal veins; 1—negative                                   |
| 4         | None                              | 80 "   | "   | " 50°C. 5 "                         | 3—ears swollen and hyperemic; 3—negative              | Left marginal ear vein                          | "                       | "                             | 3—negative   |
| 5         | 5 min.                            | 1 "  | "   | " 50°C. 5 "                         | 6—negative  | "   | "                       | "                             | 1—hemorrhage along marginal vein; 5—negative   |
| 6         | 5 "                               | 5 "  | "   | " 50°C. 5 "                         | 3—swellings   | Right marginal ear vein                         | Left marginal ear vein  | "                             | 2—diffuse hemorrhagic reaction over entire right ear; 2—doubtful; 2—negative                 |
| 7         | 5 "                               | 5 "  | "   | " 50°C. 5 "                         | 3—negative  | Left marginal ear vein                          | Right marginal ear vein | "                             | 3—negative   |
| 8         | 5 "                               | 25 "   | "   | " 50°C. 5 "                         | 3—swellings   | Right marginal ear vein                         | Left marginal ear vein  | "                             | " ; 1—died   |
| 9         | 5 "                               | 25 "   | "   | " 50°C. 5 "                         | 1—swelling; 2—negative                                | Left marginal ear vein                          | Right marginal ear vein | "                             | 2—diffuse cyanosis; 1—negative   |
| 10        | 5 "                               | 50 "   | "   | Water bath 50°C. 5 min.             | 3—swellings   | "   | "                       | "                             | 2—intense diffuse hemorrhagic reaction over entire ear; 1—negative                           |
| 11        | 5 "                               | 80 "   | "   | Bags 55°C. 5 min.                   | 3 "   | Right marginal ear vein                         | "                       | "                             | 2—intense diffuse hemorrhagic reaction over entire ear; 1—negative                           |
| 12        | 5 "                               | 50 "   | "   | "                                   | 3 "   | "   | "                       | "                             | 1—tip cyanosis (6 x 5 cm.) intense and sharply demarcated; 1—small cyanotic area; 1—negative |

\* Abbreviation Mg. 44B. designates "agar washings" filtrates of meningococcus Group III cultures.  
† Number of rabbits.

(i.e., 50 and 80 reacting units in ears exposed to 50° and 55°C.) gave intense and diffuse reactions.

It may be concluded from these experiments that preparatory intravenous injections of toxins are capable of eliciting the state of reactivity in the skin of the ear provided they are combined with temporary stasis (clamping) and thermal hyperemia.

### *Preparatory Intravenous Injections of Toxins Accompanied by Chilling*

*Protocol 1.*—The left ears of three rabbits were chilled by means of ice bags applied to both sides for a period of 10 minutes. About 2 minutes after completion of chilling, when the ears became flushed, they were clamped off at the root and 80 reacting units of meningococcus Group III (44B.) "agar washings" filtrate (T.1968) were injected into the marginal veins. The clamps were kept on for 2 minutes following the intravenous injection. 24 hours later the rabbits were injected into the marginal veins of the same ears with 25 reacting units of the above toxic filtrate. No reactions were observed.

*Protocol 2.*—In this experiment the left ears were clamped off at the base and 80 reacting units of meningococcus, Group III (44B.) "agar washings" filtrate (T.1968) were injected into the left marginal veins. Immediately after completion of the intravenous injection, ice bags were applied to the left ears. 5 minutes later the bags were removed and the clamps released. The ears appeared distinctly hyperemic shortly afterwards. 24 hours later the left marginal veins were injected intravenously with 25 reacting units, per kilo of body weight, of the same toxin. One rabbit died and the two surviving rabbits showed no reactions.

As is seen from these experiments, preparatory intravenous injections were given into the clamped ears in adequate doses. The ears were chilled prior to and following the intravenous injection for periods of 5 and 10 minutes. Distinct hyperemia followed the treatment. The injections failed, however, to elicit the state of reactivity.

### *Preparatory Intravenous Injections of Toxins in Xylol Treated Ears*

*Protocol 1.*—The left ears of six rabbits were clamped off at the base and rubbed with xylol until the veins became very prominent. Immediately afterwards, rabbits received 50 reacting units of meningococcus, Group III (44B.) "agar washings" filtrate (T.1968) into the marginal veins of treated ears. The clamps were removed 5 minutes after the intravenous injections were completed. 24 hours later the ears appeared swollen and one was slightly hemorrhagic along the marginal vein. All the rabbits received 50 reacting units of the same toxin, per kilo of body weight, into the right marginal ear veins 24 hours later. In the rabbit

# LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES

in which slight hemorrhage along the marginal vein was observed before the provocative injection, there was diffuse cyanosis with petechial hemorrhages in various parts of the ear. In another rabbit an extensive thrombosis of the marginal ear vein and its tributaries was observed. All the remaining rabbits showed no reactions.

*Protocol 2.*—The left ears of two rabbits treated with xylol until the veins became prominent were clamped off at the base. Immediately afterwards, 80 reacting units of meningococcus, Group III (44B.) "agar washings" filtrate (T.1968) were injected into the left marginal veins and the clamps removed 5 minutes later. 24 hours later, 25 reacting units, per kilo of body weight, were injected into the left marginal ear veins of these rabbits. No reactions followed.

As is seen, the provocative injection of active filtrate elicited reactions in two out of eight rabbits prepared by intravenous injection of toxin and application of xylol. In one of these, the reaction was doubtful and in another, it represented an enhancement of a primary hemorrhagic lesion following the preparatory treatment. The results of this experiment, therefore, remain inconclusive.

## *Preparatory Intravenous Injections of Toxins Combined with Ethyl Urethane, Acetylcholine, Pilocarpine Hydrochloride, Atropine, Calcium Gluconate, and Guinea Pig Liver Extract*

In this series of experiments preparatory injections were made intravenously in combination with the following substances: 10 per cent solution of ethyl urethane; 1 per cent solution of calcium gluconate; 3 per cent solution of atropine; 2 per cent solution of pilocarpine hydrochloride; acetylcholine diluted 1:500; and guinea pig liver extract in an amount of 0.85 per cent NaCl solution equal to the moist weight of liver.

The ears of the rabbits tested were clamped off at the base and the left marginal veins injected with 1 cc. each of a mixture of equal parts of 50 reacting units of meningococcus, Group III (44B.) "agar washings" filtrate (T.1968) with each of the above substances in dilutions indicated. Each mixture was tested for its preparatory effect in three rabbits. 24 hours later no reactions were observed. At this time, each of the rabbits received into the right marginal ear vein meningococcus, Group III (44B.) "agar washings" filtrate (T.1968) in a dose of 50 reacting units, per kilo of body weight. No reactions followed the provocative injections.

As is seen, preparatory intravenous injection of toxins in combination with ethyl urethane, acetylcholine, pilocarpine hydrochloride, atropine, calcium gluconate, and guinea pig liver extract failed to elicit the state of reactivity to the phenomenon under discussion.

TABLE III  
*Preparatory Intravenous Injections of Toxins in Combination with Histamine*

| Group No. | Histamine            |  | Time of clamp application | Dose of preparatory injection of toxin into left marginal ear vein | Reactions following preparatory intravenous injection | Vein used for provocative intravenous injection | Dose of provocative injection | Reactions following provocative injection               |
|-----------|----------------------|--|---------------------------|--|---|---|-------------------------------|---|
|           | Dose                 | Site                                     |                           |  |   |   |                               |   |
| 1         | 0.25 cc. dil. 1:1000 | Dermis in vicinity of left marginal vein | None                      | 40 units Mg. 44B. * T. 1968  | 3†—negative   | Right marginal ear vein                         | 50 units Mg. 44B. T. 1968     | 3†—negative   |
| 2         | "                    | "  | 5 min.                    | 28 " B. TyTL† T. 1986  | 3 "   | "   | 50 " B. TyTL T. 1986          | 3 "   |
| 3         | "                    | "  | None                      | 25 " Mg. 44B. T. 1968  | 3 "   | "   | 50 " Mg. 44B. T. 1968         | 3 "   |
| 4         | "                    | "  | 5 min.                    | 50 "   | 3—erythema and swelling                               | "   | 25 "                          | "   |
| 5         | "                    | "  | 5 "                       | 50 "   | 3—erythema  | "   | 50 "                          | 3 "   |
| 6         | "                    | "  | 5 "                       | 28 " B. TyTL T. 1986   | 3—negative  | "   | 50 " B. TyTL T. 1986          | 3 "   |
| 7         | "                    | "  | 5 "                       | 40 "   | 2—swelling; 1—negative                                | "   | 50 "                          | 3 "   |
| 8         | "                    | "  | None                      | 80 " Mg. 41B. T. 1968  | 1—tip of ear slightly hemorrhagic; 2—negative         | "   | 25 " Mg. 44B. T. 1968         | 1—definite reaction at tip (2½ x 1½ cm.); 2—negative    |
| 9         | 1 cc. dil. 1:1000    | i. v. in mixture with toxin              |                           |  |   |   |                               |   |
| 10        | "                    | "  | 5 min.                    | 20 "   | 3—negative  | "   | 25 "                          | 3—negative  |
| 11        | "                    | "  | 5 "                       | 5 "  | 3 "   | Left marginal ear vein                          | 25 "                          | 2—slight accentuation of primary hemorrhage; 1—negative |
| 12        | "                    | "  | 5 "                       | 80 "   | 2—hemorrhagic reactions; 1—negative                   | "   | 25 "                          | 3—negative  |
| 13        | "                    | "  | 5 "                       | None   | 3—negative  | "   | 25 "                          | "   |

i. v. = intravenously. Dil. = diluted.

\* Abbreviation Mg. 44B. designates "agar washings" filtrates of meningococcus Group III cultures.

† Number of rabbits.

‡ Abbreviation B. TyTL designates "agar washings" filtrates of *B. typhosus*, Strain TL cultures.

### *Preparatory Intravenous Injections of Toxins in Combination with Pituitrin*

Pituitrin in dilution 1:200 was used. In these experiments there were made preparatory intravenous injections of toxin in mixture with pituitrin or simultaneously with intradermal injections of pituitrin in the vicinity of the marginal vein. In some groups, the ears were clamped before the preparatory injection and the clamps removed 5 minutes after the injection was completed. 24 hours later the rabbits received 25 reacting units of the same toxin into the vein of clamped or non-clamped ears.

As is seen from Table IV, intradermal or intravenous injections of certain doses of pituitrin into clamped ears are prone to elicit hemorrhages. 1 hour later they may already appear along the injected vein. Approximately 24 hours later there develops cyanosis and pronounced hemorrhage which extends along the tributaries of the injected vein. These reactions become more pronounced if accompanied by intravenous injections of meningococcus, Group III (44B.) "agar washings" filtrate (T.1968). The provocative injection of toxin into veins of ears thus prepared brings about insignificant accentuation of primary reactions. It is obvious that the reactions may be interpreted as primary damage to blood vessels which are not related to the state of reactivity under discussion.

### *Preparatory Intravenous Injections of Toxins Combined with Testicular Extract*

In these experiments, there was employed rabbit testicular extract prepared according to the method described by Duran-Reynals (5).

As is seen from Table V, in Groups 1 to 4, the extract was injected into the dermis in the vicinity of the marginal veins of clamped and non-clamped ears. The preparatory injection of toxin in various doses was given intravenously immediately afterwards. The clamps were released 5 minutes after the intravenous injection was completed. The provocative injection of toxin also in various doses was given into the vein of the same or other ear 24 hours later.

The provocative injection of 50 reacting units of meningococcus, Group III (44B.) "agar washings" filtrate (T.1968) elicited definite reactions in clamped ears in two out of three rabbits prepared 24 hours previously by combined intradermal injections of testicular extracts and intravenous injections of 50 reacting units of the same filtrate (Group III). The same provocative injection failed to elicit

reactions in clamped ears prepared by combined intradermal injection of testicular extract and 25 and 80 reacting units of the filtrate (Group III), respectively.

In Groups 5 to 23, the testicular extract was mixed with the toxin in various proportions. The mixture was injected intravenously into clamped and non-clamped ears. The clamps were released 5 minutes after the intravenous injection. Provocative injections of toxins in various amounts were given into the same or other ears after different intervals of time.

In experiments of Groups 1 to 8, the interval of time between the preparatory and provocative injections was 24 hours.

In Group 7, provocative injection of 50 reacting units of *B. typhosus* T<sub>L</sub> "agar washings" filtrate (T.1968) elicited an intense reaction in one out of three rabbits prepared by intravenous injection of a mixture of undiluted testicular extract with 10 reacting units of the above filtrate. Rabbits of Group 8 prepared with 20 reacting units of the filtrate in mixture with the same amount of testicular extract showed no reactions following the provocative injection of 25 reacting units of the same filtrate. The latter experiment was carried out only with two rabbits and, therefore, remains inconclusive.

As is also seen from Table V, intense and diffuse hemorrhagic reactions followed the provocative injection of the filtrate in rabbits of Groups 9, 10, 11, 12, 14, 17, and 18. In these experiments the incubation period was short, *i.e.*,  $\frac{1}{2}$ , 1, and 2 hours; the amount of testicular extract was not less than 0.9 cc. diluted 1:4; 3 reacting units of the filtrate were used for the preparatory injection and the provocative dose varied from 2 to 25 reacting units.

Doses of three and five reacting units for preparatory and provocative injections, respectively, effective with a short incubation period, failed to elicit reactions when longer incubation periods (*i.e.*, 4, 6, and 24 hours) were allowed (Groups 19, 20, and 21).

It may be concluded from these observations that mixtures of bacterial filtrate and testicular extract are capable of inducing the state of reactivity in the rabbit's ear by way of the vascular system. The reactivity is elicited provided the circulation is interrupted for a few minutes and the amount of the filtrate is quite small. It is suggestive that the state may disappear within 4 hours following the preparation. Although the possibility of inducing the state of re-



TABLE V  
Preparatory Intravenous Injections of Toxins Combined with Testicular Extract

| Group No. | Rabbit testicular extract |  | Time of clamp application | Dose of preparatory injection of toxin into left marginal ear vein |  | Reactions following preparatory intravenous injections | Vein used for provocative intravenous injections | Dose of provocative injection |     | Time interval between preparatory and provocative injection hrs. | Reactions following provocative injection  |
|-----------|---------------------------|--|---------------------------|--|--|--|--|-------------------------------|-----|--|--|
|           | Dose                      | Site                                     |                           |  |  |  |  |                               |     |  |  |
| 1         | 0.25 cc.                  | Dermis in vicinity of left marginal vein | 5 min.                    | 25 units Mg. 44B.* T. 1968   | 11—swelling; 2—negative                | Left marginal ear vein                                 | Left marginal ear vein                           | 50 units Mg. 44B. T. 1968     | 24  | 24   | 21—negative; 1—deep cyanosis   |
| 2         | 0.25 "                    | " "                                      | None                      | " "  | 3—negative                             | " "  | " "  | 50 "                          | 24  | 24   | 3—negative venous stasis; 1—hemorrhage 4 + (1 x 1 cm.)   |
| 3         | 0.5 "                     | " "                                      | 5 min.                    | 50 "   | 2                                      | Right marginal ear vein                                | " "  | 50 "                          | 24  | 24   | 2—no reactions; 1—died   |
| 4         | 0.5 "                     | " "                                      | 5 "                       | 80 "   | 3                                      | " "  | " "  | 50 "                          | 24  | 24   | 3—negative   |
| 5         | 1 cc. dil. 1:5            | Left marginal ear vein                   | 5 "                       | None   | 2—negative; 2—doubtful hemorrhage      | " "  | " "  | 50 " B. TyTL† T. 1986         | 24  | 24   | 3  |
| 6         | 1 cc.                     | " "                                      | 5 "                       | "  | 3—erythema                             | Left marginal ear vein                                 | " "  | 50 " Mg. 44B. T. 1968         | 24  | 24   | 2—negative; 1—diffuse hemorrhage 3 + 2—slight erythema   |
| 7         | 0.9 "                     | " "                                      | 5 "                       | 10 units B. TyTL T. 1986   | 2—slight erythema                      | " "  | " "  | 50 " B. TyTL T. 1986          | 24  | 24   | 1—negative; 1—hemorrhage 4 + (7 x 1½). Petechial hemorrhage over entire ear; 1—diffuse petechial hemorrhage over ear |
| 8         | 0.9 cc. dil. 1:4          | " "                                      | 5 "                       | 20 "   | 2—negative; 1—slight hemorrhage at tip | " "  | " "  | 25 "                          | 1/2 | 1/2  |  |
| 9         | "                         | " "                                      | "                         | 3 "  | "                                      | " "  | " "  | 5 "                           |     |  |  |

|    |                   |   |   |   |   |   |   |   |  |   |                         |    |   |   |    |  |
|----|-------------------|---|---|---|---|---|---|---|--|---|-------------------------|----|---|---|----|--|
| 10 | 0.9 cc.           | " | " | 5 | 3 | " | " | " | 2—negative                               | " | "                       | 5  | " | " | 1  | 1—hemorrhage 2+ tip of ear; 1—slight erythema                                      |
| 11 | 0.9 cc. dil. 1:4  | " | " | 5 | 3 | " | " | " | 3  | " | "                       | 25 | " | " | 1  | 1—negative; 2—extensive hemorrhage 2+  |
| 12 | "                 | " | " | 5 | 3 | " | " | " | 1—erythema 4+; 2—slight erythema         | " | "                       | 5  | " | " | 2  | 2—negative; 1—hemorrhage 4+ (8½ x 6 cm.)   |
| 13 | 0.9 cc.           | " | " | 5 | 3 | " | " | " | 3—negative                               | " | "                       | 15 | " | " | 2  | 3—negative   |
| 14 | 0.9 cc. dil. 1:4  | " | " | 5 | 3 | " | " | " | 1—negative; 1—doubtful hemorrhage at tip | " | "                       | 2  | " | " | 2  | 1—negative; 1—hemorrhage 4+ (4 x 4 cm.)  |
| 15 | "                 | " | " | 5 | 1 | " | " | " | 3—negative                               | " | "                       | 5  | " | " | 2  | 3—negative   |
| 16 | 0.9 cc. dil. 1:10 | " | " | 5 | 3 | " | " | " | 3  | " | "                       | 5  | " | " | 2  | 3  |
| 17 | 0.9 cc. dil. 1:4  | " | " | 5 | 3 | " | " | " | 3  | " | "                       | 25 | " | " | 2  | 2—died; 1—hemorrhage 4+ (2½ x 2 cm.) and diffuse petechial hemorrhage in both ears |
| 18 | "                 | " | " | 5 | 3 | " | " | " | 3  | " | Right marginal ear vein | 15 | " | " | 2  | 1—diffuse hemorrhagic reaction along inner border of ear                           |
| 19 | "                 | " | " | 5 | 3 | " | " | " | 3  | " | Left marginal ear vein  | 5  | " | " | 4  | 3—negative   |
| 20 | "                 | " | " | 5 | 3 | " | " | " | 3  | " | "                       | 5  | " | " | 6  | 3  |
| 21 | "                 | " | " | 5 | 3 | " | " | " | 3  | " | "                       | 5  | " | " | 24 | 3  |

Dil. = diluted.

\* Abbreviation Mg. 44B. designates "agar washings" filtrates of meningococcus, Group III cultures.

† Number of rabbits.

‡ Abbreviation B. TyT<sub>L</sub> designates "agar washings" filtrates of *B. typhosus*, Strain TL cultures.

activity is obvious from the experiments cited, the exact conditions of its reproduction should be considered with a great deal of reserve. Apparently, there exist individual fluctuations in susceptibility of rabbits which may serve as a source of error. These fluctuations are illustrated by the unexpectedly negative results of Group 13. The negative results of Group 16 were possibly due to a high dilution of testicular extract (*i.e.*, 0.9 cc. diluted 1:10), and those of Group 15 to the use of only 1 reacting unit for preparation.

If the assumption be granted that the state of reactivity induced by way of the vascular system with the aid of testicular extract is of short duration, it becomes clear why larger provocative doses are necessary when the interval of time is longer. Thus, positive results were obtained in Group 7 where as many as 50 reacting units were used for the provocative injection 24 hours after preparation. Rabbits of Group 21, yielding negative results, received only 5 reacting units for the provocative injection after the same interval of time.

#### RÉSUMÉ AND COMMENTS

In the experiments recorded in this paper, attempts were made to determine whether local reactivity to bacterial filtrates could be elicited by way of the vascular system. Preparatory injections of bacterial filtrates of ascertained skin-preparatory potency were given into the marginal ear vein. In the greater portion of the experiments the circulation of the ear was interrupted by application of a clamp for short periods of time prior to and following the preparatory injection of filtrates. In most of the experiments injections of filtrates were given intravenously in mixture with agents intended to modify capillary and tissue permeability. In the remaining experiments, the preparatory intravenous injections of filtrates were accompanied by simultaneous intradermal injections of some of these agents. Various intervals of time between preparatory and intravenous injections were allowed in the experiments with testicular extract. The following was observed.

The state of reactivity could not be elicited by preparatory intravenous injections of adequate doses of bacterial filtrates alone into clamped and non-clamped ears. The state also failed to appear in combination with cold, xylol, ethyl urethane, acetylcholine, pilocar-

pine hydrochloride, atropine, calcium gluconate, guinea pig liver extract, histamine dihydrochloride, adrenalin chloride, and pituitrin. However, preparatory intravenous injections of toxins were capable of eliciting the state of reactivity in the rabbit's ear when they were accompanied by a thermal hyperemia produced by exposure to 45°, 50°, and 55°C. It was also possible to induce a state of reactivity of short duration when a mixture of the preparatory factors with testicular extract was given into the veins of clamped ears. In some experiments provocative injections of small doses of a filtrate given  $\frac{1}{2}$ , 1, and 2 hours following the preparatory injections with such a mixture, elicited severe and diffuse reactions. It is noteworthy that in these experiments the incubation period and the duration of the reactivity was considerably shorter than that following the intradermal preparatory injection. In the latter, at least 8 hours of incubation period are required and it may last as long as 96 hours (6).

It is difficult to interpret the observation that heat and testicular extract were the only agents which allowed the preparation of the rabbit's ear by way of the vascular system whilst numerous substances employed which are capable of influencing profoundly the capillary permeability failed to do so. According to Duran-Reynals (7), McClean (8), and Favilli (9), the Reynals factors are capable of producing striking and immediate increase in dermal permeability which lasts for 24 hours. The well known rapid spreading of testicular extract in the injected site is due to an extreme dilatation of the capillaries and lymph spaces. It is obvious that when an intradermal injection of a bacterial filtrate is made for the purpose of elicitation of the state of reactivity of the phenomenon under discussion, a perivascular depot of the injected material is formed which comes into continuous contact with the cells and blood vessels of the site injected. The fact that an incubation period of at least 8 hours is required for the elicitation of the state of reactivity may be due to relative impermeability of the cells which does not permit a rapid entrance of the preparatory factors into them. The rôle of the testicular extract and heat accompanying the intravascular preparation may, then, be twofold; *i.e.*, to allow the passage of the injected substances into the surrounding tissues through a rapid increase in the capillary permeability; and to enhance the cell permeability. The latter assumption is based

on the fact that a considerably shorter incubation period is necessary for preparation with mixtures of the preparatory factors with testicular extract injected intravenously than when the preparatory factors alone are injected intradermally.<sup>1</sup>

In 1924, Sanarelli (10) described experiments in which rabbits received an intravenous injection of a sublethal dose of a live culture of cholera vibrio followed by an intravenous injection of *B. coli* or *B. proteus* culture filtrate, 24 hours later. The second injection elicited hemorrhagic lesions in the small intestines, mesentery, and kidneys, and killed a large percentage of rabbits. Invariably it was possible to demonstrate cholera vibrio in the intestinal wall of animals injected. Combined injections of heat killed or autolyzed cholera vibrio with *B. coli* or *B. proteus* culture filtrates produced no effect. The reverse order of injections, i.e., *B. coli* culture filtrate followed by injection of live cholera vibrio culture, also gave little effect. Sanarelli concluded from his experiments that there exists a definite selective affinity of cholera vibrio for the intestines; that the reactions described are anaphylactic in nature (*epithalaxic*) requiring sensitization with live cholera vibrio; and that the experimental picture obtained is pathognomonic of human cholera.

After the author of this paper described the phenomenon of local skin reactivity to bacterial filtrates in 1928, Gratia and Linz interpreted Sanarelli's observation in the light of the latter phenomenon. They assumed that the first intravenous injection of the live cholera vibrio induced in the intestines a state of reactivity by means of the preparatory factors operative in the phenomenon of local skin reactivity to bacterial filtrates. The provocative injection of the filtrate of *B. coli* or *B. proteus* elicited, then, hemorrhagic lesions in the intestines. To prove this contention, Gratia and Linz (2) gave to guinea pigs two intravenous injections of bacterial filtrates potent in the elicitation of the phenomenon of local skin reactivity (cholera vibrio), 24 hours apart and obtained hemorrhages in the peritoneal cavity and, in one instance, hemorrhagic lesions in the large intestines subsequently to the second injection.

Phenomenon of general reactivity to *B. coli* culture filtrates was later studied

<sup>1</sup>The effect of testicular extract upon the phenomenon under discussion was previously studied by Duran-Reynals by means of experiments somewhat different from those described in this paper. Rabbits were either prepared by intradermal injection of a mixture of testicular extract with bacterial filtrate, or received an intravenous injection of testicular extract 24 hours after the intradermal preparatory injection of bacterial filtrate. Following the intravenous injection of bacterial filtrate there was observed spreading of the lesion accompanied by a definite reduction in intensity. The author concluded that testicular extract did not enhance the susceptibility to the phenomenon of local skin reactivity to bacterial filtrates.

again by Gratia and Linz (2) and Apitz (11). Gerber (12) in these laboratories, recently studied this phenomenon with *B. typhosus* and meningococcus "agar washings" filtrates. These authors gave repeated intravenous injections of filtrates potent in the elicitation of the phenomenon of local skin reactivity to bacterial filtrates. In most of the experiments there were given two intravenous injections 24 hours apart. Diffuse vascular lesions were observed in the liver, spleen, kidneys, adrenals, pancreas, bone marrow, and lungs. Apitz and Gerber made extensive gross and histologic studies. No intestinal lesions were noted by them.

Gratia and Linz (2), Dienes (13), Bordet (14), Apitz (11), Freund (15), and Koplik (16) in these laboratories, observed hemorrhagic lesions at the sites of tuberculous and other bacterial and virus infections following an intravenous injection of certain potent heterologous bacterial filtrates.

Thus, there is in the literature a group of observations apparently related to each other. In the light of experiments described in this paper, an attempt will be made to point out in a form of a working hypothesis possible differences in essential mechanisms of the above observations.

In the phenomenon of local skin reactivity to bacterial filtrates the state of reactivity is elicited through the influence of certain soluble bacterial factors introduced into the perivascular tissue. The provocative intravenous injection of suitable material produces lesions in the capillary network, especially in the small veins and venules, and subsequently in the tissues themselves. No arterial damage is observed.

In the phenomenon of general reactivity to bacterial filtrates (*i.e.*, two intravenous injections, 24 hours apart, of bacterial filtrates potent in the elicitation of the phenomenon of local skin reactivity) diffuse vascular lesions occur in organs above mentioned. As in the phenomenon of local skin reactivity, there is observed damage in the capillary network, small veins, and venules. It was shown in the work on the rabbit's ear that preparation by way of the vascular system takes place provided agents capable of enhancing capillary permeability and possibly cell permeability are employed. In experiments on general reactivity, when the state of reactivity is elicited by way of the vascular system in the absence of any auxiliary agents increasing the vascular permeability, it is reasonable to expect that the physiologic differences of capillary permeability of organs and especially varia-

on the fact that a considerably shorter incubation period is necessary for preparation with mixtures of the preparatory factors with testicular extract injected intravenously than when the preparatory factors alone are injected intradermally.<sup>1</sup>

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As noted before, sites of spontaneous or induced infections may possess the state of reactivity of the phenomenon under discussion. For this reason the possible effect of bacterial filtrates upon pre-existing foci in the experimental animals is to be considered in the experiments on the phenomenon of general reactivity to bacterial filtrates.

Freund (19) recently observed that hemorrhagic reactions may appear at sites injected with silver nitrate in tuberculous guinea pigs following intravenous injection of potent heterologous bacterial filtrates. In such experiments the inflammatory reaction set up by silver nitrate may be able to localize bacteria and bacterial toxic substances circulating in the vascular system of the infected tuberculous animal. This possibility is clearly postulated by the work of Opie (20), Menkin (21), Cannon and Pacheco (22), and others. Returning to Freund's observations, the localization from the blood stream of certain factors secreted by the bacteria during the course of the active infection may now induce a state of reactivity at the site of fixation. Bacterial factors capable of eliciting the phenomenon of local skin reactivity were recently shown to exist in tuberculous cultures (Shwartzman (23)). Obviously, subsequent provocative injection of bacterial filtrates could produce a reaction at the site of the silver nitrate injection. It is noteworthy in this connection that silver nitrate alone (*i.e.*, without the presence of active infection in the animal) has no skin-preparatory potency (Shwartzman (6)). This mechanism is not implied necessarily as one operative in the experiments by Freund. An attempt is made, as in other instances, to outline the complex interference of additional agents in studies on the phenomena of general and local skin reactivity to bacterial filtrates. Data reported in this paper concerning the elicitation of the state of reactivity by way of the vascular system make these considerations necessary in the evaluation of the various facts reported in the literature.

Following intravenous injections into clamped ears of mixtures of pituitrin, adrenalin chloride, and histamine dihydrochloride with bacterial filtrates, primary hemorrhagic and thrombotic lesions were observed. These lesions were rarely enhanced by the provocative injections of potent filtrates. Possibly, similar effects were obtained by Marcus and Schmidt-Weyland. These authors, quoted by Rössle

tions in the venous supply, may condition the occurrence of lesions in different organs. Rous (17) in collaboration with F. Smith, Hudack, and McMaster, showed clearly that there exist essential differences in the permeability of capillaries and venules based on structural features. It is of interest in this connection that the lesions of the phenomenon of general reactivity occur in organs in which a high degree of capillary permeability exists under physiologic conditions (kidney, liver, spleen, bone marrow, etc.); and also that they take place mostly in the venules. Further proof is also brought by the finding of Stolyghwo (18) that the factors operative in the phenomenon of local skin reactivity are excreted by the kidneys of rabbits injected intravenously with bacterial filtrates and also of typhoid fever patients. The phenomenon of general reactivity to bacterial filtrates may, then, be identical with the phenomenon of lesions in normal animals is conditioned by the vascular permeability of various organs.

Next, then, one should consider the additional rôle of general reactivity, and in the fact that the distribution of lesions in normal animals is conditioned by the vascular permeability of various organs. Next, then, one should consider the additional rôle of general reactivity, and in the fact that the distribution of lesions in normal animals is conditioned by the vascular permeability of various organs. Next, then, one should consider the additional rôle of general reactivity, and in the fact that the distribution of lesions in normal animals is conditioned by the vascular permeability of various organs.

capable of modifying the vascular and tissue permeability, and thus being indirectly responsible for the elicitation of the state of general reactivity. Sanarelli's observation belongs to this group in which, in addition to soluble bacterial filtrates, the effects of the live organisms used for the preparatory injection are to be considered. These effects are possibly as follows:

Local inflammatory reactions to live bacteria which may change the capillary permeability; formation of bacterial foci which may act as provocative agents upon distant reactive areas; and the inherent selective affinity of the organisms employed for various organs, etc. With our present knowledge, an attempt is made merely to outline roughly the possible complex rôle that the combined effect of live bacteria with the soluble bacterial factors may have in the elicitation of the lesions described by Sanarelli. It is noteworthy in this connection that in his experiments, reactions were observed in the small intestines whilst no intestinal lesions were observed by Apitz and Gerber in the phenomenon of general reactivity to bacterial factors alone; and that live cholera vibrio was invariably isolated from the intestinal wall.

GREGORY SHWARTZMAN

observations concerning the elicitation of the phenomena of organ reactivity by means of live bacterial cultures and the filtrates thereof.

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- ### EXPLANATION OF PLATES
- PLATE 26
- Micrococcus filtrate

EXPLANATION OF PLATES  
PLATE 26

FIG. 1. Phenomenon of local skin reactivity to meningococcus filtrate in the rabbit ear. (Meningococcus, Group III (44B.) "agar washings" filtrate (T.1968).) 0.25 cc. diluted 1:2 was injected intradermally and 100 reacting units were in-

(24),<sup>2</sup> gave repeated injections of adrenalin to rabbits previously treated with bacterial toxins and, in some instances, they obtained gangrene and thrombosis.

It is of interest that in spite of the primary hemorrhagic lesions obtained with mixtures above described, the state of reactivity did not take place. This fact may serve as an additional argument in favor of the contention that the ability of bacterial factors to induce the state of reactivity to the phenomenon under discussion is entirely independent of primary inflammatory and hemorrhagic effects in otherwise normal animals.

Experiments are under way to determine the effect of bacterial invaders and various agents capable of changing the capillary permeability, upon the phenomenon of general reactivity to bacterial filtrates.

#### SUMMARY

The skin of the rabbit's ear is considerably more resistant than the abdominal skin to the phenomenon of local skin reactivity to bacterial filtrates. Ten times the provocative dose is required if it is given into the vein of the prepared ear and thirty times the provocative dose if it is given into the vein of the non-prepared ear.

The state of reactivity cannot be elicited by a preparatory intravenous injection of bacterial filtrates alone into clamped and non-clamped ears. The state also fails to appear in combination with cold, xylol, ethyl urethane, pilocarpine hydrochloride, atropine, calcium gluconate, guinea pig liver extract, histamine dihydrochloride, adrenalin chloride, and pituitrin.

Preparatory intravenous injections of toxins are capable of eliciting the state of reactivity in the rabbit's ear when they are accompanied by thermal hyperemia (*i.e.*, exposure to 45°, 50°, and 55°C.). It is also possible to induce the state of reactivity when a mixture of the preparatory factors with testicular extract is given into the veins of clamped ears. The incubation period required may be less than 2 hours.

In the light of the above experiments, there are discussed various  
<sup>2</sup>Unfortunately, the reference to the original paper describing these experiments was not available.



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(Stewartman: Local skin reactivity to bacterial filtrate.)









# THE COLONY MORPHOLOGY OF TUBERCLE BACILLI

## III. THE RELATION BETWEEN VIRULENCE AND COLONY FORM

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PLATE 28

(Received for publication, July 9, 1935)

Although it is a well recognized fact among bacteriologists that many bacterial species decline rather quickly in virulence during artificial cultivation, it is also well known that some strains of tubercle bacilli have retained a considerable virulence during many years of cultivation on artificial media. However, it has not infrequently been observed (1) that some strains of tubercle bacilli also exhibit a gradual loss of pathogenic properties. Except possibly in the case of BCG (2, 3), efforts to restore the virulence of these attenuated strains have not been successful, nor is it known what factors are responsible for the *in vitro* attenuation of the organisms. Experiments were therefore planned to determine whether the virulence of various bovine and human strains bore any relationship to the morphology of colonies isolated from animals inoculated with them.

Since the autumn of 1933, we have isolated a number of new strains of tubercle bacilli from human sources. Also during this interval we have received a human strain<sup>1</sup> designated "Jamaica;" nine bovine strains<sup>2</sup> isolated between April, 1928, and January, 1933; and one bovine strain<sup>3</sup> isolated by Ravenel. These, together with our stock human Strain H-37 and bovine Strain B-1, comprising a total of eleven bovine strains, and eight strains of human origin, were employed in comparative tests of virulence. Each strain was reisolated from the test animals and studies were made of its cultural

<sup>1</sup> This strain was received from Dr. J. Freund of the Cornell University Medical School, New York.

<sup>2</sup> These strains were received from the late Dr. Theobald Smith.

<sup>3</sup> We received this strain from The Henry Phipps Institute, Philadelphia.



TABLE I  
*Source, Date of Isolation, Number of Cultural Passages, and Previous Knowledge of Virulence of Nineteen Strains of Tubercle Bacilli Used for Virulence Tests*

| Strain of T.B. | Isolated by      | Isolated from           | Date of isolation     | No. of cultural passages before inoculation | Last previous observation of virulence         |
|----------------|------------------|-------------------------|-----------------------|---|--|
| II-37          | Baldwin, Saranac | Human sputum            | 1905                  | Innumerable                                 | Moderate                                       |
| Jamaica        | Freund           | Human tracheal node     | Fall, 1933            | 4   | Moderate (Freund)                              |
| MR             | Smithburn        | Human psoas abscess     | Dec., 1933            | 5   | Moderate                                       |
| 3103           | Smithburn        | <i>M. rhesus</i> spleen | Jan., 1934            | 3   | Undetermined                                   |
| 3104           | Smithburn        | Human knee biopsy       | Jan., 1934            | 4   | Undetermined                                   |
| Thompson       | Smithburn        | Human psoas abscess     | Feb., 1934            | 3   | Undetermined                                   |
| Bell           | Thomas           | Human sputum            | Reisolated Feb., 1934 | 2   | Fully virulent                                 |
| Kilty          | Smithburn        | Human wrist fluid       | Feb., 1934            | 3   | Undetermined                                   |
| B-1            | Baldwin, Saranac |                         | 1904                  | Innumerable                                 | Very low                                       |
| Bov. 32        | Theobald Smith   | Cow                     | Apr., 1928            | Unknown                                     | Fully virulent when isolated. Not since tested |
| Bov. 33        | Theobald Smith   | Cow                     | May, 1928             | Unknown                                     |  |
| Bov. 34        | Theobald Smith   | Cow                     | Apr., 1929            | Unknown                                     |  |
| Bov. 35        | Theobald Smith   | Cow                     | Apr., 1929            | Unknown                                     |  |
| Bov. 36        | Theobald Smith   | Cow                     | Apr., 1929            | Unknown                                     |  |
| Bov. 37        | Theobald Smith   | Cow                     | Oct., 1929            | Unknown                                     |  |
| Bov. 38        | Theobald Smith   | Cow                     | Mar., 1932            | Unknown                                     |  |
| Bov. 39        | Theobald Smith   | Cow                     | Oct., 1932            | Unknown                                     |  |
| Bov. 40        | Theobald Smith   | Cow                     | Jan., 1933            | Unknown                                     |  |
| Bov. Ravenel   | Ravenel          |                         |                       | Unknown                                     | Marked   |

and morphologic characteristics, with special reference to the phenomenon of dissociation. The studies to be reported were, therefore, of a twofold nature: pathological and bacteriological.

*Procedures*—The classic work of Theobald Smith (4-6) in differentiation of human from bovine type tubercle bacilli by animal inoculation left little to be learned from the standpoint of comparative pathology. However, in order to classify each of our strains, it was necessary to inoculate both rabbits and guinea pigs with each culture. Eleven bovine strains and eight of human origin were used in the experiments. Table I shows the source of each strain, date of isolation, and, when available, the number of cultural passages prior to inoculation.

In each instance a considerable quantity of bacterial growth was removed from the stock culture, weighed in the moist condition, and suspended in sterile saline by grinding in a mortar. Dilutions of the bacterial suspensions were then prepared so that 1 cc. contained 0.1 mg. of organisms (approximately 5,000,000 bacteria). Each animal received 0.1 mg. of bacteria; rabbits were inoculated intravenously, guinea pigs subcutaneously in the right groin.

The rabbits used for inoculation were Lilac hybrid stock bred at The Rockefeller Institute; they were of approximately the same age and each weighed about 2,000 gm. The guinea pigs were from open market stock and each weighed about 250 to 300 gm. each. All animals, with but a few exceptions, were females. Two rabbits and two guinea pigs were inoculated with each bacterial suspension, thirty-eight rabbits and thirty-eight guinea pigs being used for the tests of the nineteen strains. All of the animals were kept under identical conditions as regards light, temperature, ventilation, and diet. They were weighed at weekly intervals. 30 days after inoculation half the animals receiving each strain of organism were killed, the rabbits by intravenous injection of air, the guinea pigs with chloroform. All survivors were killed about 60 days after inoculation. The spleen or bone marrow of each animal was removed aseptically and cultures were made. (The method of making cultures and the colony characteristics will be discussed later.) A complete autopsy was performed on each animal and comparisons were made of the gross pathology present. Sections of the fixed tissues were stained with hematoxylin and eosin; and sections of the lung, bone marrow, and spleen were stained for tubercle bacilli with hematoxylin and anilin fuchsin.

*Compilation of Data on Extent of Lesions*—Direct comparisons were made at autopsy of the extent of lesions produced by the various strains of tubercle bacilli. Three to five blocks of lung, two bone marrows, eight or nine lymph nodes (including superficial and visceral), and the visceral organs from each animal were examined microscopically. Records were made regarding the size of lesions and the number of lesions in all viscera and bone marrows. Lymphadenopathy was evaluated + to + + + +, according to the portion of the node involved. The sum of these data gave a figure for each animal which represented quite accurately the extent of tuberculous lesions.

the remaining bovine strains, Dr. Theobald Smith stated that the virulence of each had been tested at the time of isolation and that

TABLE II  
*Relative Extent of Lesions Produced in Inoculated Animals by Eight Strains of Tubercle Bacilli of Human Origin*

| By strain | After days | Extent of tuberculous lesions produced |         |
|-----------|------------|--|---------|
|           |            | Total lesions                          |         |
|           |            | Guinea pigs                            | Rabbits |
| 3104      | 30         | 26.5                                   |         |
|           | 60         | 38.0                                   | 19.0    |
| Bell      | 30         |  | 0.5     |
|           | 60         | 28.0                                   |         |
| H-37      | 30         | 34.0*                                  | 11.0    |
|           | 60         |  | 9.0     |
| 3103      | 30         | 25.0                                   |         |
|           | 60         | 36.0                                   | 26.0    |
| MR        | 30         |  | 11.0    |
|           | 60         | 24.0                                   |         |
| Thompson  | 30         | 36.0                                   | 9.0     |
|           | 60         |  | 3.5     |
| Jamaica   | 30         | 17.0                                   |         |
|           | 60         | 38.0                                   | 10.0    |
| Kilty     | 30         | 21.0                                   | 0.5     |
|           | 60         | 30.0                                   |         |
|           | 30         |  | 21.0    |
|           | 60         | 19.0                                   | 7.0     |
|           | 30         | 32.0                                   | 16.0    |
|           | 60         |  | 1.0     |
|           | 30         | 22.0                                   |         |
|           | 60         | 18.5                                   | 23.0    |
|           |            |  | 10.0    |

The figures represent units of tuberculous lesions and were obtained by macroscopic and microscopic comparisons of the tissues. Actual numbers of lesions not indicated; the data are entirely relative.

Decimals indicate a minute tuberculous focus in some viscus.

\* Died 49 days.

there were no marked differences. In the results to be discussed, however, it will be shown that there were remarkable differences in virulence of these strains. In addition to the B-1 strain, bovine

*Studies on Virulence*

*Strains of Human Origin.*—Each of the strains of tubercle bacilli of human origin, except the H-37, was recently isolated. The Jamaica strain was isolated from a Jamaican native by Dr. J. Freund. The remainder were isolated in this laboratory. Only moderate differences in pathogenic properties of these organisms for guinea pigs were noted, although some of them produced much more extensive, albeit regressive, lesions in rabbits. Most extensive lesions in guinea pigs were produced by the strain designated 3104, although both the H-37 and Thompson strains produced more extensive lesions in rabbits (30 days). Each of the strains of human origin produced macroscopic lesions in rabbits at 30 days; these lesions, after 60 days, were in every instance regressive. In guinea pigs but one strain produced lesions less extensive at 60 than at 30 days, namely, the Kilty strain. For reasons to be discussed later, this may with certainty be regarded as a bovine strain of but moderate virulence. Table II shows the relative extent of lesions produced by these human strains in rabbits and guinea pigs examined 30 and 60 days after inoculation.

In Table II the eight strains of human origin are arranged from above downward in the order of diminishing lesions produced by each in guinea pigs. It will be noted that there is no relationship between the extent of lesions which a strain produces in guinea pigs and the extent of lesions produced in rabbits. The table shows, however, that in each instance the lesions in rabbits are less extensive at 60 days than at 30 days; in guinea pigs, however, all strains except Kilty induced progressive lesions. It will be shown later that the latter is a bovine strain.

It may be stated at this point that none of the animals inoculated with human strains showed appreciable weight loss. This was in all probability due to the relatively short duration of the experiments and comparatively small inoculum. (The inoculating dose used by Theobald Smith to produce wasting disease was much larger.)

*Bovine Strains.*—The B-1 strain used in these experiments has suffered a sharp decline in virulence in our hands during the past 5 years. The Ravenel strain, which we have had for about 18 months, is a strain possessed of marked pathogenic properties. Regarding

TABLE III

*Relative Extent of Lesions Produced in Inoculated Animals by Eleven Strains of Bovine Tubercle Bacilli*

| Extent of tuberculous lesions produced |            |               |         |
|--|------------|---------------|---------|
| By strain                              | After days | Total lesions |         |
|  |            | Guinea pigs   | Rabbits |
| Bov. 36                                | 30         | 26*           | 33      |
|  | 60         | 44†           | 37‡     |
| Bov. 39                                | 30         | 20.5          | 38      |
|  | 60         | 39§           | 33      |
| Bov. 38                                | 30         | 23            | 34**    |
|  | 60         | 33            | 43††    |
| Bov. Ravenel                           | 30         | 21            | 29‡‡    |
|  | 60         | 32            | 30§§    |
| Bov. 40                                | 30         | 21            | 16      |
|  | 60         | 29            | 28      |
| Bov. 34                                | 30         | 15            | 10      |
|  | 60         | 34            | 17.5    |
| Bov. 33                                | 30         | 13            | 22      |
|  | 60         | 13            | 14.5    |
| Bov. 37                                | 30         | 8.5           | 5       |
|  | 60         | 21            | 17      |
| Bov. 32                                | 30         | 6.5           | 1.5     |
|  | 60         | 9.5           | 1.5     |
| B-1                                    | 30         | 5             | 1       |
|  | 60         | 3             | 0.5     |
| Bov. 35                                | 30         | 5.5           | 0.5     |
|  | 60         | 0.5           | 0.5     |

The figures represent units of tuberculous lesions observed macroscopically and microscopically. The data are relative and do not indicate actual number of lesions. Decimals indicate a minute focus in some viscus.

\* Died 24 days.

† Killed 35 days.

‡ Died 28 days.

§ Died 60 days.

|| Died 35 days.

\*\* Died 31 days.

†† Died 40 days.

‡‡ Died 31 days.

§§ Died 35 days.



Strains 32 and 35 proved to be practically avirulent; bovine Strains 33 and 37 were of moderate virulence; bovine Strain 34 was slightly more virulent, whereas Strains 36, 38, 39, 40, and Ravenel were each highly virulent. Since Strains 32 to 40 were each found to be fully virulent when isolated, it is therefore definite that some of them had declined and that in general the more recently isolated strains were more virulent than those strains undergoing prolonged artificial cultivation. The irregularity with which such changes in pathogenic properties may occur is illustrated by the fact that bovine Strain 36, isolated in April, 1929, is considerably more virulent than Strain 35, Strain 40, isolated about the same time, and somewhat more virulent than and 40 each produced far more extensive wasting and rapidly fatal disease than Strains B-1, 32, and 33.

Table III shows the extent of lesions produced in guinea pigs and rabbits each inoculated with one of the eleven bovine strains. It will be noted from Table III that several animals inoculated with bovine strains died before the experiment was terminated. Two or four animals inoculated with Strains 36, 38, and Ravenel died, each in 40 days or less, whereas two animals inoculated with Strain 39 died at 35 and 60 days respectively. From the standpoint of fatal termination and extent of lesions, therefore, these four strains, 36, 38, 39, and Ravenel, may be considered as most virulent, whereas Strains B-1, 32, and 35 were least. It may also be remarked that animals inoculated with the virulent strains lost weight, although wasting did not occur in animals inoculated with the less virulent strains.

It may be seen also from Table III that the lesions produced by Strains B-1, 33, and 35 were static or retrogressive and did not progress. This result may be compared with that obtained with the Kilty strain (Table II). In fact the animal responses to Strains Kilty and bovine 33 were quite similar. It would appear, therefore, that the Kilty organism is of bovine type and of but moderate virulence. This view has been confirmed by serologic methods which will be discussed in a subsequent publication.

Chart 1 shows the rate of progression or regression of tuberculosis in the rabbits and guinea pigs each inoculated with one of seven virulent human strains or one of the seven bovine strains of tubercle

each animal. The results were recorded as 0 to +++. The data pertaining to the three sections each from the four animals inoculated with one strain were added; the figures representing these sums are recorded in Table IV. In the table the seven human and twelve bovine type strains are arranged horizontally in order of diminishing virulence from left to right. Beneath the name or number of each

TABLE IV

*The Relation between Virulence and Number of Acid-Fast Bacilli in Lungs, Spleen, and Bone Marrow*

| Human T.B. strain.....                  | 3104 | Bell | H-37 | 3103 | MR | Thompson | Jamaica |
|---|------|------|------|------|----|----------|---------|
| Order of virulence.....                 | 1    | 2    | 3    | 4    | 5  | 6        | 7       |
| *Bacilli in lesions in guinea pigs..... | 10   | 9    | 2    | 3    | 3  | 2        | 0       |
| Bacilli in lesions in rabbits.....      | 1    | 1    | 3†   | 1    | 0  | 0        | 0       |
| Bacilli in lesions, total...            | 11   | 10   | 5    | 4    | 3  | 2        | 0       |

| Bovine T.B. Strain.....                | 36 | 39 | 38 | Ravenel | 40 | 34 | Kilty | 33 | 37 | 32 | 1  | 35 |
|--|----|----|----|---------|----|----|-------|----|----|----|----|----|
| Order of virulence.....                | 1  | 2  | 3  | 4       | 5  | 6  | 7     | 8  | 9  | 10 | 11 | 12 |
| Bacilli in lesions in guinea pigs..... | 12 | 11 | 9  | 5       | 9  | 6  | 2     | 0  | 3  | 0  | 0  | 0  |
| Bacilli in lesions in rabbits.....     | 18 | 14 | 13 | 14      | 12 | 1  | 2     | 3  | 1  | 0  | 0  | 0  |
| Bacilli in lesions, total...           | 30 | 25 | 22 | 19      | 21 | 7  | 4     | 3  | 4  | 0  | 0  | 0  |

\* The number of bacilli is relative in each instance. Manner of obtaining data is explained in text.

† Of rabbits killed 60 days after inoculation with human strains, only one (with H-37) showed acid-fast bacilli in lesions.

strain are recorded the numerical order of virulence and the relative number of acid-fast bacilli in the tissues of four animals inoculated with each strain. The data for rabbits and for guinea pigs are also given separately.

It will be seen from the data in Table IV that there is a very definite relation between the number of organisms in the lesions and the virulence of the strain. The strains of greater virulence show the greater

bacilli which proved to be fully virulent. The human strains included were all those in Table II except Kilty. The bovine strains included were: 34, 36, 37, 38, 39, 40, and Ravenel.

These observations make it clear that in order to classify a given mammalian strain, the virulence of which has not been recently tested, it is necessary to inoculate both rabbits and guinea pigs. A human strain of full virulence produces progressive tuberculosis in guinea pigs but not in rabbits. A bovine strain of full virulence produces progressive tuberculosis in both rabbits and guinea pigs, whereas bovine strains of low virulence produce retrogressive lesions in both rabbits and guinea pigs. Therefore, if a strain of mammalian

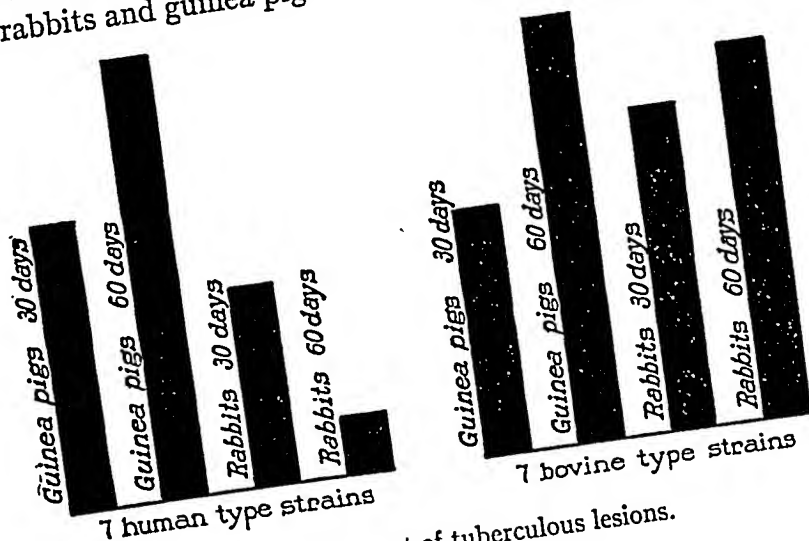


CHART 1. Extent of tuberculous lesions.

tubercle bacilli be inoculated into rabbits, and if it produces lesions which regress, it does not follow that it is a human type bacillus unless it be shown also that the organism produces progressive tuberculosis in guinea pigs.

*Acid-Fast Bacilli in the Lesions.*—The evaluation of pathogenic properties of the various human and bovine strains, and their arrangement in Tables II and III in order of virulence were made on the basis of extent of lesions in the animals inoculated with each strain. Both the number and size of lesions were considered. In addition to the study of the lesions, surveys were made to determine the relative numbers of acid-fast bacilli in the lungs, spleen, and bone marrow of

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each animal. The results were recorded as 0 to +++. The data pertaining to the three sections each from the four animals inoculated with one strain were added; the figures representing these sums are recorded in Table IV. In the table the seven human and twelve bovine type strains are arranged horizontally in order of diminishing virulence from left to right. Beneath the name or number of each

TABLE IV  
*The Relation between Virulence and Number of Acid-Fast Bacilli in Lungs, Spleen, and Bone Marrow*

|   | 3104 | Bell | H-37 | 3103 | MR | Thompson | Jamaica |
|---|------|------|------|------|----|----------|---------|
| Human T.B. strain.....                  |      |      |      |      |    |          |         |
| Order of virulence.....                 | 1    | 2    | 3    | 4    | 5  | 6        | 7       |
| *Bacilli in lesions in guinea pigs..... | 10   | 9    | 2    | 3    | 3  | 2        | 0       |
| Bacilli in lesions in rabbits.....      | 1    | 1    | 3†   | 1    | 0  | 0        | 0       |
| Bacilli in lesions, total...            | 11   | 10   | 5    | 4    | 3  | 2        | 0       |

|  | 36 | 39 | 38 | Ravenel | 40 | 34 | Kilty | 33 | 37 | 32 | 1  | 35 |
|--|----|----|----|---------|----|----|-------|----|----|----|----|----|
| Bovine T.B. Strain.....                |    |    |    |         |    |    |       |    |    |    |    |    |
| Order of virulence.....                | 1  | 2  | 3  | 4       | 5  | 6  | 7     | 8  | 9  | 10 | 11 | 12 |
| Bacilli in lesions in guinea pigs..... | 12 | 11 | 9  | 5       | 9  | 6  | 2     | 0  | 3  | 0  | 0  | 0  |
| Bacilli in lesions in rabbits.....     | 18 | 14 | 13 | 14      | 12 | 1  | 2     | 3  | 1  | 0  | 0  | 0  |
| Bacilli in lesions, total...           | 30 | 25 | 22 | 19      | 21 | 7  | 4     | 3  | 4  | 0  | 0  | 0  |

\* The number of bacilli is relative in each instance. Manner of obtaining data is explained in text.

† Of rabbits killed 60 days after inoculation with human strains, only one (with H-37) showed acid-fast bacilli in lesions.

strain are recorded the numerical order of virulence and the relative number of acid-fast bacilli in the tissues of four animals inoculated with each strain. The data for rabbits and for guinea pigs are also given separately.

It will be seen from the data in Table IV that there is a very definite relation between the number of organisms in the lesions and the virulence of the strain. The strains of greater virulence show the greater

numbers of bacilli and the strains of least virulence show the fewest (or none). The correlation is by no means mathematically perfect but the estimates cannot be reduced to a mathematical basis. Lewis and Sanderson (7) suggested that the number of organisms remaining in the lesions may be an expression of their rate of multiplication. Strength will be added to this view later, when it will be shown that there is a definite relation between the number of organisms present in stained sections and the number of colonies which develop when cultures are made from the tissues. In other words, most of the stained organisms must be viable and a large number of them therefore indicates that multiplication has taken place.

It will also be noted from the data in Table IV that the animals inoculated with certain of the bovine strains showed a great many more organisms in their lesions than any of the animals inoculated with human type tubercle bacilli. This was largely due to apparent failure of the human type strains to multiply in the rabbit host. The rabbits killed 30 days after inoculation with human strains showed relatively few organisms and of those killed 2 months after inoculation, only one animal showed acid-fast bacilli in the tissues. Among animals inoculated with bovine strains, however, the rabbits showed as many (or more) organisms in their tissues as the guinea pigs inoculated with the same strains. Guinea pigs inoculated with virulent bovine bacilli showed approximately the same number of bacilli in their lesions as guinea pigs inoculated with equally virulent bacilli of human type.

#### *Cultural Studies*

The bacteriological studies to be discussed here were concerned with efforts to determine any possible relationship of the virulence of a given strain of tubercle bacilli to the colony morphology of the organisms recovered from inoculated animals. Petroff (8) and his associates had already demonstrated differences in virulence dependent upon the colony form of organisms inoculated. Furthermore, we (9) were able to show that under proper cultural conditions smooth, rounded, shiny colonies of tubercle bacilli were obtained which resembled the smooth forms of other pathogenic bacteria. However, the questions to which we sought answer in the present experiments

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were: Do strains of tubercle bacilli of low virulence produce smooth colonies, and if so, are the smooth colonies produced in as great number as in the case of highly virulent strains?

Certain aspects of the cultural characteristics of the tubercle bacilli used in these experiments were discussed previously (9),<sup>4</sup> at which time it was found that the use of  $H_2SO_4$  or NaOH in isolating the organisms inhibited the development of smooth colonies. The technical aspects of the experiments were given fully and will be repeated here but briefly.

*Procedures.*—Animals used in the tests of virulence were killed with chloroform or by intravenous injection of air. Cultures were made from animals sacrificed 30 and 60 days after inoculation with human strains, and from animals sacrificed 30 days after inoculation with bovine strains. Cultures were not reisolated from animals inoculated with bovine strains and allowed to survive beyond 30 days. At autopsy the spleen or bone marrow was removed aseptically, suspended in sterile saline, and the suspension was divided into three portions. One portion was treated with  $H_2SO_4$  (6 per cent by weight), another with NaOH (3 per cent by weight), and the third was untreated. The treated portions were neutralized after 12 to 22 minutes. Slants of culture media (chiefly Petroff's gentian violet egg medium and Corper's egg yolk-glycerine medium) were then seeded with each of the three tissue suspensions from each animal. The tubes were sealed with paraffin and incubated at 37°C. They were examined periodically with a binocular dissecting microscope, the final examination being made after 4 months' incubation. At each examination data were recorded as to the approximate number of colonies on each slant and the per cent of various types of colonies present. In some instances colonies at first smooth, later became granular. The results to be discussed are therefore only those recorded after 4 months' incubation.

*Human Strains.*—In the cultures recovered from animals inoculated with human strains there was no correlation between the number of organisms seen in the stained sections and the number of positive cultures or number of colonies recovered from the tissues. However, it was noted that smooth, rough, and intermediate colonies, similar to those previously described and illustrated (9), occurred in cultures of each human strain. The smooth colonies were most numerous in the cultures from guinea pigs. In cultures recovered from the spleens of rabbits 30 days after inoculation with human strains, the colonies which developed (few in number) showed pro-

<sup>4</sup> Smithburn (9), page 400.

nounced granular characteristics. Cultures made from the tissues of rabbits 60 days after inoculation were consistently negative. It appears, therefore, that the rabbit possesses the capacity to alter the human type organisms so that they have the characteristics of the granular forms, and then the organisms are in some manner destroyed. The failure to recover the human organism from the spleens of rabbits was recorded by Lurie (10), but the capacity of the animal host to influence colony characteristics of the organism has not to our knowledge been observed. This power to alter and destroy the human tubercle bacillus is not inherent in the guinea pig. The cultures recovered from these animals at 30 or 60 days showed numerous smooth colonies, and the total growth was often more luxuriant in cultures from animals allowed to survive 2 months. Lurie (10) also found that bovine tubercle bacilli remained viable in the tissues of rabbits until the death of the host. It would seem, therefore, that a susceptible host has not the capacity to inhibit multiplication of the organisms or to destroy them, whereas the naturally resistant host can do so.

*Bovine Strains.*—It was shown in the preceding section of this report that among the bovine strains of bacilli employed, there were very marked differences in virulence. Some strains were highly pathogenic, others avirulent, and between these extremes were certain strains possessed of moderate pathogenic properties. This broad range made possible studies which could not be done with the human strains, among which the gradations of pathogenesis were slight. With the bovine strains it was possible to establish definite correlations between: (1) virulence and per cent of positive cultures; (2) virulence and number of colonies recovered. These data and number and per cent of smooth colonies recovered. These data are presented in tabular form. Table V shows the number and per cent of positive cultures isolated from animals, each inoculated with one of six bovine strains of high or one of six bovine strains of low virulence. 80 per cent of the cultures from animals inoculated with highly virulent strains were positive, whereas only 20 per cent of the cultures from animals inoculated with strains of low virulence were positive.

These data are further analyzed to show the approximate number

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of colonies recovered from the animals inoculated with each strain. Table V shows that not only were more positive cultures recovered from the animals inoculated with strains of high virulence, but that the number of colonies per tube was greater and the total number of colonies recovered was about 25 times as great as in the case of cul-

TABLE V  
Number and Per Cent of Positive Cultures and Number of Colonies Recovered from Animals Inoculated with Bovine Tubercle Bacilli of High or Low Virulence

| Strain of bovine T.B. | No. of animals cultured | No. of cultures | Cultures positive No. | Cultures positive per cent | Approximate No. of colonies | Average No. of colonies per culture | Virulence |
|-----------------------|-------------------------|-----------------|-----------------------|----------------------------|-----------------------------|-------------------------------------|-----------|
| 36                    | 2                       | 15              | 13                    | 86.6                       | 4,900                       | 209                                 | High      |
| 39                    | 2                       | 15              | 12                    | 80.0                       | 2,504                       |                                     |           |
| 38                    | 3                       | 18              | 12                    | 66.6                       | 421                         |                                     |           |
| Ravenel               | 2                       | 11              | 9                     | 81.8                       | 2,411                       |                                     |           |
| 40                    | 2                       | 15              | 14                    | 93.4                       | 3,161                       |                                     |           |
| 34                    | 2                       | 15              | 12                    | 80.0                       | 1,705                       | 31                                  | Low       |
| Total....             | 13                      | 89              | 72                    | 80.89                      | 15,102                      |                                     |           |
| Kilty                 | 4                       | 27              | 4                     | 14.8                       | 20                          |                                     |           |
| 33                    | 2                       | 15              | 3                     | 20.0                       | 205                         |                                     |           |
| 37                    | 2                       | 15              | 5                     | 33.3                       | 180                         |                                     |           |
| 32                    | 2                       | 15              | 3                     | 20.0                       | 30                          | 659                                 |           |
| 1                     | 2                       | 15              | 6                     | 40.0                       | 224                         |                                     |           |
| 35                    | 2                       | 15              | 0                     | 0                          | 0                           | 20.58                               |           |
| Total....             | 14                      | 102             | 21                    | 20.58                      | 659                         |                                     |           |

Approximate numbers of colonies determined as follows: Growth on each tube was recorded as scant (1 to 5 colonies), fair (6 to 20 colonies), good (21 to 100 colonies), or excellent (101 to 1,000 colonies). The actual number of colonies present in many of the cultures was recorded. In others colony counts were done on portions of the culture and approximations of the whole number were made. The total number of colonies per strain of organisms (Table V) is the sum of data on all cultures recovered from animals inoculated with that strain.

tures recovered from animals inoculated with strains of low pathogenicity.

While studying the cultures recovered from the animals, it was noted that three principal types of colonies occurred on almost every slant. These were: (a) rounded, non-granular, glossy colonies with



entire margin (designated smooth, Figs. 1, 2, and 3); (b) flat, spreading, stippled, or finely granular colonies (designated intermediate, Fig. 2); and (c) coarsely granular colonies of irregular contour, rising sharply from the medium but adhering to its surface (designated rough, Fig. 2). These three colony variants are illustrated in Fig. 2, a photograph of a culture recovered from a rabbit, R 3459<sup>s</sup> inoculated with bovine Strain 39. Records were made of the approximate

TABLE VI  
*Number of Each of Three Colony Variants Recovered from Animals Inoculated with Bovine Type Strains of High or Low Virulence*

| Strain of bovine T.B. | No. of colonies recovered |              |       | Virulence |
|-----------------------|---------------------------|--------------|-------|-----------|
|                       | Smooth                    | Intermediate | Rough |           |
| 36                    | 3,110                     | 1,790        | 0     | High      |
| 39                    | 1,534                     | 941          | 29    |           |
| 38                    | 5                         | 399          | 17    |           |
| Ravenel               | 56                        | 1,674        | 681   |           |
| 40                    | 343                       | 2,806        | 12    |           |
| 34                    | 1,034                     | 591          | 80    |           |
| Total.....            | 6,082                     | 8,201        | 819   |           |
| Per cent.....         | 40.27                     | 54.3         | 5.43  |           |
| Kilty                 | 5                         | 15           | 0     | Low       |
| 33                    | 18                        | 122          | 65    |           |
| 37                    | 24                        | 146          | 10    |           |
| 32                    | 0                         | 20           | 10    |           |
| 1                     | 24                        | 196          | 4     |           |
| 35                    | 0                         | 0            | 0     |           |
| Total.....            | 71                        | 499          | 89    |           |
| Per cent.....         | 10.77                     | 75.72        | 13.5  |           |

per cent of each of the three colony variants in each culture. The percentage values multiplied by the whole number of colonies on the slant gave the relative numbers of each of the three variants. A summation of these data is presented in Table VI.

The data presented in Table VI show that a large per cent of colonies were smooth when recovered from animals inoculated with <sup>s</sup> These are serial numbers of animals used in this laboratory during a period of years.

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strains of high virulence. In cultures recovered from animals inoculated with the less virulent strains, there were also smooth colonies but in much smaller number, whereas there were relatively greater numbers of intermediate and rough colonies. It is therefore clear that dissociation did occur in strains of low virulence (Strain B-1, Table VI, is avirulent), but it is also clear that there was a definite relation between the virulence of a strain, the ease with which it could be recovered, the number of colonies which were obtained, and the per cent of colonies having the smooth form.

#### DISCUSSION

In any consideration of bacterial virulence, at least two factors must be recognized; namely, the powers of resistance of the host, and the capacity of the microorganisms to survive, multiply, and carry on their metabolic processes within the host. Two variables are therefore present in any experiments designed to determine the pathogenic properties of a bacterium. Regarding the pathogenic properties of tubercle bacilli, certain facts are already well known, both as pertains to factors of host resistance and to properties of the bacteria. The factors of host resistance were first brought to light by Theobald Smith, and then elaborated upon by subsequent workers. In addition to the variations in resistance of different animal species, there are rather marked differences among animals of the same species. In order to evaluate factors concerned with the bacteria, it is therefore necessary to reduce to a minimum the variation of host factors by careful selection of animals. Likewise it is essential in comparing the virulence of several strains to control the size of the inoculating dose in so far as this is possible.

Giving due consideration to the above named factors, all possible precautions were taken in these experiments. Nevertheless, it is probable that factors beyond control influence the course of disease in certain animals, and that slight differences in pathogenic properties between two strains are not highly significant. It is therefore probably beyond the limits of absolute accuracy to place each of a large number of strains of bacteria in its proper order of virulence without employing a very large number of animals and allowing prolonged periods of time to elapse to determine whether less virulent

strains eventually produce progressive disease. But with the methods employed here, gross differences in pathogenic properties of various strains do become apparent, and between the strains of greatest and those of least virulence there is the difference of 50 per cent mortality in 60 days on the one hand, and almost complete absence of demonstrable lesions on the other.

On the basis of our observations, the statement seems justified that, among recently isolated strains of human tubercle bacilli, there are moderate but definite differences in pathogenic properties. Since each of the human type strains was of high or moderate virulence, there were no marked differences in cultural characteristics of the organisms reisolated from inoculated animals. It was noted, however, that cultures recovered from rabbits 30 days after inoculation of human strains showed more marked granular characteristics than cultures similarly recovered from guinea pigs. And all efforts to recover the human type organism from rabbits 60 days after inoculation were unsuccessful. In view of this it seems that the defensive mechanism of the rabbit successfully depresses those characteristics associated with virulence and after a time succeeds in destroying the bacteria.

In the guinea pigs none of the human strains caused appreciable loss of weight, or death, within the time limits of the experiments. This probably was due to the relatively small dose inoculated, the additional fact that the animals used were young and growing, and to insufficient lapse of time. However, inoculation of each of the human type strains into guinea pigs was associated with lesions considerably more extensive at 60 than at 30 days.

The virulence of bovine strains was approximately the same for rabbits as for guinea pigs. That is, if a given strain produced rapidly progressive, wasting fatal disease in rabbits, the result in guinea pigs was similar. But if another strain produced in rabbits lesions which progressed for a time and then slowly regressed, the result in guinea pigs was almost invariably the same. Among the twelve bovine type strains studied (one, Kilty, isolated from man), the virulence ranged from very high to very low. Although no hard and fast rule can be established, it is in general true that the more recently isolated strains were the more virulent, and the strains cultivated *in vitro* for a long time were less virulent, indicating slow attenuation.

To our knowledge, it has not been shown that tubercle bacilli killed *in vitro* by heat or by chemical methods possess staining properties at all different from those of the living organism. To the latter statement an exception may be drawn in the case of certain organic solvents which remove certain lipoids with which the property of acid-fastness is associated. This fact would indicate that acid-fast bacteria which appear in stained sections of animal tissue might be either viable or dead. Certain of our results, however, speak against this view, particularly in the case of the bovine type organisms. When large numbers of acid-fast bacilli were found in the tissues, large numbers were also recovered in culture; and when the number in the tissue was small, or when there were none, few or no organisms were recovered in cultures. This would make it seem that most, if not all the organisms to be seen in tissues are viable, and contrarily that once the bacillus is dead it soon loses its acid-fastness and bacillary form.

Very large numbers of bovine type bacilli were recovered from animals inoculated with the most virulent strains; from animals inoculated with the less virulent strains, fewer and fewer organisms were recovered, and from animals with an avirulent strain, no organisms were obtained. This seems to indicate in the first instance a capacity of virulent tubercle bacilli to multiply in the body of the host, and in the other instance a capacity of the host to destroy or eliminate less virulent bacteria.

With exceptions in the case of but three strains of organisms, all cultures reisolated from animals inoculated with bovine type bacilli showed three types of colonies. And although the data were not given in this report, the same was true of five of the seven human strains. All positive cultures, either of human or bovine type strains, showed either two or three colony variants. These variants are described and illustrated and have been previously discussed (9). In cultures of highly virulent bovine strains, the smooth variant is present in far greater numbers than in cultures of strains of low virulence. In case of the rough variant, however, there are relatively greater numbers in cultures of strains of low virulence than in cultures of more virulent strains. Thus, there is a definite relationship between the virulence of a given strain, the number of organisms to be seen in tissues of animals 30 or 60 days after inoculation with that

strain, the number of organisms appearing in cultures from tissues of inoculated animals, and the prevalence of smooth colonies in these cultures.

Although a small number of smooth colonies did occur in cultures of strains possessing little virulence, it need not necessarily follow that reinoculation of these variants would cause progressive disease. Another experience, not reported here, may clarify this point. A smooth variant of an avian tubercle bacillus, A-1, with which many experiments have been done in this laboratory, especially by Dr. J. T. Geiger, was formerly highly virulent for rabbits and fowl. This strain gradually became attenuated and is now completely avirulent. Its colony morphology has not changed and colonies of this strain are indistinguishable from those of another smooth, highly virulent, avian strain in our possession. It therefore appears that dissociation of tubercle bacilli may occur, irrespective of virulence, but it is also true in all probability that of the variants of a virulent strain the smooth form possesses greatest pathogenicity.

Further studies of certain factors which influence dissociation of tubercle bacilli and of the significance of smooth colonies in strains of low virulence will be reported later.

#### SUMMARY

All the strains of human tubercle bacilli described in this report as recently isolated from clinical cases proved to be virulent and showed but slight differences in pathogenic properties. Neither did they show extreme variation in virulence when compared with Strain H-37, isolated many years ago. Between twelve bovine strains cultivated *in vitro* for varying periods, there was a much wider range in virulence, some being so attenuated as to give regressive lesions in both rabbits and guinea pigs, while other strains were highly pathogenic. In general, the more recently isolated strains were the more virulent. Attenuation occurs with prolonged *in vitro* cultivation, but the rate of attenuation is apparently not the same for all strains.

Studies of cultures recovered from inoculated animals demonstrated that in the case of bovine tubercle bacilli virulence is correlated with three phenomena: the number of bacilli which can be stained in tissues of inoculated animals, the number of organisms recoverable in

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cultures from the tissues, and the proportion of smooth colonies in these cultures. All cultures of either human or bovine type recovered from animals showed either two or three types of colonies. In general the percentage of the smooth form varied directly with virulence. However, some smooth colonies were present in strains having little or no pathogenicity. One example was cited of an avian strain which had lost its virulence while retaining the smooth colony form—indicating that, although pathogenicity is usually correlated with smooth colony form, it is not necessarily so. And smooth variants devoid of virulence do occur.

In the comparison of the effect of the same human strain on rabbits and guinea pigs, it was shown that native resistance (of the rabbit) was associated with a power to dissociate the inoculated bacilli into a greater proportion of rough forms, and then to destroy them. This power is not possessed by the naturally susceptible animal (guinea pig).

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## EXPLANATION OF PLATE 28

FIG. 1. Smooth colonies of bovine Strain 40 reisolated from the spleen of Rabbit R 3463, killed 31 days after inoculation. Age of culture 119 days.  $\times 8.5$ .

FIG. 2. Smooth (A), intermediate (B), and rough (C) colonies of bovine Strain 39, reisolated from the spleen of Rabbit R 3459, killed 31 days after inoculation. Note veil-like border of the intermediate colonies and irregular contour of the rough variant. Age of culture 119 days.  $\times 8.5$ .

FIG. 3. Smooth colonies of bovine Strain 36 recovered from the spleen of Rabbit R 3447, killed 32 days after inoculation. Age of culture 119 days. Note the absence of any but rounded, glistening colonies.  $\times 8.5$ .





1. Single tubercle bacillus.

2. (Smith) Group C morphology of tubercle bacilli. (H.D.)





# THE COMPARATIVE BEHAVIOR OF MAMMALIAN EGGS IN VIVO AND IN VITRO

## I. THE ACTIVATION OF OVARIAN EGGS\*

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PLATES 29 AND 30

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The eggs of most mammals are shed from the ovary with the first polar body formed. The mechanism controlling this stage of maturation has never been investigated in detail. Furthermore, under normal conditions only shed ova are fertilized. Does this indicate that the first maturation division is an essential prelude to fertilization? Or may ovarian eggs in fact be activated before the first meiotic division?

This investigation concerns itself with these problems, and falls into two parts dealing with: (1) the mechanism controlling the first meiotic division; (2) the capacity for fertilization of ovarian eggs. Superficially unrelated, these two studies are aspects of the broad problem of the fundamental nature of the activation process.

### EXPERIMENTAL

The rabbit is especially favorable material for this study since it ovulates only after copulation. It has been established that copulation results in a stimulation of pituitary secretion, and that the amount of anterior pituitary secretion necessary to induce ovulation occurs during the 1st hour after copulation (Deansley, Fee, and Parkes, 1930). The injection of pituitary extracts or of prolactin induces ovulation (Friedman, 1929); and furthermore, ovulation induced by stimulating hormones occurs at 10 hours after injection (Bellerby, 1929). Ova are normally shed with the first polar body at 10 hours after copulation. According to Heape (1905) both polar bodies are formed at 9 hours after copulation.

Heape's statement is but partially correct. Only one polar body is formed. We have investigated this situation in detail, and our data are summarized in Table I. Before copulation occurs the ovum contains a single large vesicular nucleus about 30 microns in diameter (Fig. 1). At 2 hours after copulation some

\* This investigation has been aided by a grant from the National Research Council Committee for Problems of Sex.

of the ripe ova show signs of the initiation of maturation. The diakinesis-like chromatin begins to condense into tetrads, but the nuclear membrane remains intact (Fig. 2). The separation of strands of follicle cells adjacent to the corona radiata begins to be manifest. By 4 hours after copulation the tetrads of the first polar spindle are formed and the nuclear membrane is ordinarily dissolved (Figs.

TABLE I  
*Progressive Changes in Maturing Ovarian Eggs during the Time Interval between Copulation and Ovulation*

| Time elapsed since copulation | No. of cases observed | Condition of the egg  | Condition of the follicles  |
|-------------------------------|-----------------------|---|---|
| hrs.<br>0                     | 10                    | Egg fully grown. Nucleus vesicular, in some cases vesicular tetrads present                                   | Average diameter 970 $\mu$ . The follicular epithelium forms a spider web. In some cases the egg is in a cumulus                        |
| 2                             | 9                     | The vesicular nucleus present in most cases. In all cases tetrads present                                     | Spider web arrangement of granulosal. Liquor folliculi increasing in amount. Disintegration of follicle cells adjacent to corona begins |
| 4                             | 7                     | Vesicular membranes have disappeared. Only traces present. Tetrads free in cytoplasm                          | Average diameter 1045 $\mu$ . Follicular epithelium changing from spider web to cumulus type  |
| 6                             | 6                     | Chromatic material decreases very much in size and forms the first spindle. No trace of nuclear membrane left | Most follicles in cumulus type. The liquor folliculi becomes increasingly viscous   |
| 7                             | 4                     | First polar body extruded in many cases. The remaining chromatin moves sideways                               | As in preceding type  |
| 8                             | 10                    | First polar body present in all cases   | Average diameter 1125 $\mu$   |
| 9                             | 4                     | First polar body present. Second spindle in place and ready to form the second polar body                     | Average diameter 1310 $\mu$ . Eggs almost free in follicle  |

3, 4, and 5). The metaphase plate is found in all maturing ova by 6 hours after copulation, and the freeing of the egg and corona from the connecting follicular strands is almost complete. The first polar body is given off between 7 and 8 hours after copulation (Fig. 6). The second polar spindle is formed during the 9th hour post coitum and the ripe ovum (Fig. 7) is shed between 9½ and 10½ hours.

There is also a definite follicular enlargement during this period, our measurements of fixed material giving a maximum follicular diameter of 970 microns (average of 10 follicles) before copulation and an increase to 1125 microns (average of 10 follicles) by the 8th hour after copulation.

When pregnancy urine, antuitrin-S,<sup>1</sup> or saline pituitary extracts, are injected intravenously, exactly the same sequence of events ensues.

We attempted to determine whether the maturation process involved in the production of the first polar body was due to the direct action of pituitary hormones.

Ova were taken from the large follicles of unmated does and cultivated *in vitro*. The culture medium consisted of sterile rabbit blood plasma, to which was added in the control series, several drops of a phosphate-buffered Ringer's solution. In the experimental series we substituted for the Ringer's solution extracts of beef pituitary glands made in Ringer's or a preparation of maturity hormone.<sup>2</sup> The ova were rapidly dissected in a Ringer-serum solution, care being taken to remove the viscous liquor folliculi that often surrounds the cumulus oöphorus. The cumulus cells were not dissected away to any extent, since it seemed desirable to reduce handling to a minimum. The ova were cultured for varying periods at 38°C. in hollow ground slides sealed with paraffin, fixed in Bouin's solution, and prepared for microscopic examination.

The data of this experiment are summarized in Table II. It is evident that the control series differs in no way from the experimental series. All the cultured ova had formed tetrads and in some cases the nuclear membrane had dissolved. In certain instances the nucleus resembled the fusion nucleus of normally fertilized eggs (Fig. 8), as though chromosome division had taken place without polar body formation but with subsequent refusion of the nuclear elements.

These data indicate that polar body formation as a result of direct stimulation by pituitary hormones is improbable. It is possible however, that the hormone concentration *in vitro* was too low to effect any stimulation of the ova. In order to test this we determined the effect of varying concentrations of the two preparations injected intravenously. The data are summarized in Table III.

It will be seen that a dosage of  $\frac{1}{4}$  cc. of maturity hormones was sufficient to cause polar body formation, and 2 cc. of Ringer's extract

<sup>1</sup> We are indebted to Dr. Oliver Kamm of Parke, Davis & Co. for the antuitrin-S.

<sup>2</sup> This preparation was supplied to us by Dr. J. B. Collip of McGill University, to whom we express our gratitude.

of beef pituitary was similarly effective. Slightly less than this minimal dosage was added to the cultures receiving the maximum amount of hormone. If we consider that the minimum effective dosage *in vivo* is distributed throughout the organism and probably partitioned or excreted in such a manner as to make available to the ovary only a fraction of the amount injected, it seems entirely probable that a sufficient amount was available to the cultured ova.

TABLE II  
Data on Ovarian Eggs Obtained by Puncture of Graafian Follicles, and Cultured in  
(a) Media Containing Pituitary Hormones, (b) Media of Ringer-Locke Solution

| Time of culturing | No. of cultures | Medium                                  | Results   |
|-------------------|-----------------|---|---|
| min.              |                 |   |   |
| 20                | 14              | Ringer-Locke + 1 drop beef pituitary    | Vesicular tetrads formed in all cases   |
| hrs.              |                 |   |   |
| 2                 | 11              | Ringer-Locke + 2 drops beef pituitary   | In some cases vesicular tetrads and some free tetrads were formed. Some formed polar bodies |
|                   |                 |   | Vesicular tetrads in all cases, except 3 which had free tetrads                             |
| 24                | 9               | Ringer-Locke + 1 drop maturity hormone  | Vesicular tetrads in all cases  |
|                   |                 |   |   |
|                   | 4               | Ringer-Locke + 2 drops maturity hormone | Vesicular tetrads, free tetrads, structures resembling fusion nuclei                        |
| 25                |                 |   | Vesicular tetrads and free tetrads  |
|                   | 7               | Ringer-Locke + 3 drops maturity hormone | Free tetrads  |
| 25                |                 |   | Rudiment of first polar spindle   |
|                   | 18              | Ringer-Locke                            | Vesicular tetrads, free tetrads, fusion nuclei  |
| 2                 | 3               | "                                       |   |
| 4                 | 3               | "                                       |   |
| 6                 |                 |   |   |
| 20                | 16              |   |   |

Since direct action of the pituitary hormone upon the ova seems to be excluded, the probability exists that a second hormone acts directly upon the eggs, and that a stimulating concentration of this second hormone occurs as the result of pituitary hormone activity. Pituitary secretions are known to be thyreotropic as well as gonadotropic, and accordingly thyroxin activity may be involved. Moreover, Carter (1932) has demonstrated that thyroxin-like substances are probably concerned in the activation of sea urchin eggs. We therefore injected thyroxin intravenously, as indicated in Table IV.

Ovulation in no instance occurred, but a definite cytological effect was observed. This consisted of an atresia of ovarian follicles pre-

TABLE III

*Effect of Injecting Varying Concentrations of Saline Beef Pituitary Extract and of Maturity Hormone upon the Maturation of Ovarian Eggs*

| Preparation used for injection | No. of animals | Method of injection              | Time between injection and autopsy | Results  |
|--------------------------------|----------------|----------------------------------|------------------------------------|--|
|                                |                |                                  | hrs.                               |  |
| ½ cc. beef pituitary           | 1              | Subcutaneous, unmated doe        | 12                                 | No ovulation, maturation of ovarian eggs                               |
| 1 " " "                        | 2              | Intravenous, 6 hrs. after mating | 6                                  | Normal ovulation not disturbed   |
| 1½ " " "                       | 2              | Intravenous, unmated doe         | 12                                 | Ovulation  |
| 2 " " "                        | 2              | Intravenous, 6 hrs. after mating | 4                                  | Normal ovulation not disturbed. A second wave of maturation in ovaries |
| 2½ " " "                       | 1              | Subcutaneous, unmated doe        | 12                                 | No ovulation, many ovarian eggs matured, some formed polar bodies      |
| 4 " " "                        | 2              | Intravenous, 6 hrs. after mating | 16                                 | Double ovulation   |
| 5 " " "                        | 2              | Subcutaneous, unmated doe        | 12                                 | Ovulation  |
| ¼ " maturity hormone           | 1              | Intravenous, unmated doe         | 14                                 | No ovulation, ovarian eggs maturing                                    |
| ½ " " "                        | 1              | Intravenous, unmated doe         | 14                                 | " "  |
| ½ " " "                        | 1              | Intravenous, unmated doe         | 12                                 | No ovulation, ovarian eggs mature                                      |
| 1 " " "                        | 2              | Subcutaneous, unmated doe        | 14                                 | Ovulation  |
| 2 " " "                        | 1              | Subcutaneous, unmated doe        | 14                                 | Superoovulation  |

ceded or accompanied by the formation of chromosome tetrads in the ova of large follicles (Fig. 9). In a few instances polar body formation

occurred but ordinarily not at 8 hours after injection of the thyroxin but some time later. The effect of thyroxin injection is therefore not as rapid as the pituitary-induced effect, nor, under the conditions of these experiments, as complete. Eggs cultured *in vitro* with crystalline thyroxin added to the medium (see Table V) formed polar bodies

TABLE IV  
*Effect of Injecting Thyroxin\* or Thyroprotein† of Varying Doses upon the Maturation of Ovarian Eggs*

| Preparation used for injection | No. of animals used | Method of injecting              | Time between injection and autopsy | Results  |
|--------------------------------|---------------------|----------------------------------|------------------------------------|--|
|                                |                     |                                  | hrs.                               |  |
| 1 cc. thyroxin                 | 1                   | 1 dose, intravenously            | 12                                 | No ovulation, slight atresia, vesicular nuclei                                   |
| 2 " "                          | 1                   | 2 doses, intravenously           | 14                                 | No ovulation, tetrads and polar bodies in ovarian eggs                           |
| 3 " "                          | 2                   | 3 doses, intravenously           | 12                                 | No ovulation, varying degree of maturation of ovarian eggs, atresia, large cysts |
| 4 " "                          | 2                   | 4 doses, intravenously           | 14                                 | As in preceding case   |
| 1 " thyroprotein               | 2                   | 1 dose, intravenously, mated doe | 24                                 | Normal ovulation. Widespread atresia in ovaries and various maturation phenomena |
| 2 " "                          | 2                   | 2 doses, intravenously mated doe | 24                                 | As in preceding case   |

\* 10 mg. crystalline thyroxin + 1 drop of 4 per cent NaOH + 1 cc. H<sub>2</sub>O.  
Dosage given in one injection  $\frac{1}{10}$  cc.

† Parke, Davis & Co. preparation No. 30, each cubic centimeter containing 0.00125 gm.

but so did the control ova cultured without added thyroxin. In one instance an ovum cultured with thyroxin formed a second polar body. With this one exception there is no detectable cytological difference between ova cultured in thyroxin-containing media and the control ova.

All of the *in vitro* experiments indicate that the mere process of explanting ova results in the initiation of maturation. This implies that the nutritive conditions that make for maturation *in vivo* are automatically duplicated *in vitro*. Pituitary hormones (and to a lesser extent thyroxin) must induce, in the follicle, these nutritive conditions. What these conditions are is not entirely clear, but it is obvious that *in vivo* the pituitary hormones initiate the events leading to their establishment.

In another experiment ovarian eggs were handled and cultured in Ringer's solution alone (Table II) to obviate the possibility that the blood plasma might

TABLE V

*Effect of Adding Thyroxin\* to Culture Media upon the Maturation of Ovarian Eggs in Culture*

| Composition of the medium              | No. of cultures made | Time interval of culturing | Result  |
|--|----------------------|----------------------------|---|
|  |                      | <i>hrs.</i>                |   |
| Plasma + 1 drop thyroxin               | 6                    | 24                         | All cultures showed about the same phenomena which included tetrad formation in all cultured eggs. In some of the cultures polar bodies formed, or the vesicular membrane dissolved |
| " + 3 drops "                          | 4                    | 22                         |   |
| " + 4 " "                              | 3                    | 22                         |   |
| " + 6 " "                              | 8                    | 24                         |   |
| " + 8 " "                              | 4                    | 24                         |   |
| " + 2 " Ringer-Locke solution          | 22                   | 20-24                      |   |
| Plasma + 6 drops Ringer-Locke solution | 8                    | 20-24                      |   |

\* The thyroxin solution was made up as described in Table IV.

contain sufficient pituitary hormone or thyroxin to stimulate maturation *per se*. Maturation spindles were formed in these ova whether maturity hormone was present or not. Finally, motion pictures were taken of single ovarian eggs cultured in Ringer's solution. The dissolution of the nuclear membrane and the formation of tetrads and the polar spindle occurred at the same rate as *in vivo*.

When ripe ova are removed from the follicles of unmated does and placed in sperm suspensions, sperm penetration occurs (Figs. 10 and 11), and in some instances the male pronucleus forms (Fig. 12). The first polar body is given off by these ova just as in control ova cultured without sperm, but control ova (Table II) never give off the



second polar body whereas a number of these ova with sperm do (see Table VI).

Ovarian ova inseminated *in vitro* have been transplanted into the fallopian tubes of pseudopregnant rabbit does. 2 to 3 days later these ova were recovered from the tubes and examined. In a small number regular cleavage had taken place. In others cleavage was irregular or absent. The latter cases are undoubtedly due to a condition of polyspermy. It is difficult to avoid polyspermy with *in vitro* inseminations, since it is necessary to have sperm suspensions

TABLE VI  
*Results of Inseminating Ovarian Eggs in Culture with Sperm Suspensions*

| Approximate age of egg    | Condition of sperm | Time of culture | Result   |
|---------------------------|--------------------|-----------------|--|
| 0 hrs., from unmated does | Undiluted          | min. 20         | Sperm penetration takes place in most cases. Polyspermy is frequent. Male pronuclei may form. Polar bodies are not always present. The order of the process may become upset, for instance tetrads and a male pronucleus being present in the same egg |
| " "                       | "                  | hrs. 2          |  |
| " "                       | "                  | 4               |  |
| " "                       | "                  | 6               |  |
| " "                       | "                  | 7               |  |
| " "                       | "                  | 8               |  |
| " "                       | Diluted 1:4        | 16-24           | The same phenomena as in the upper series, but development seems to proceed further and with greater regularity. Second polar bodies are formed in many cases, especially in older eggs. The heavy sperm suspension leads to frequent polyspermy       |
| " "                       | " 1:10             |                 |  |
| 2 hrs. after mating       | Undiluted          | 26              |  |
| 4 " " "                   | "                  | 19              |  |
| 6 " " "                   | "                  | 20              |  |
| 7 " " "                   | "                  | 20              |  |
| 8 " " "                   | "                  | 20              |  |

sufficiently concentrated to insure sperm entry and not so dilute that the sperm will die before any entry is possible. Even with fairly dilute suspensions of active sperm we have found two or more spermatozoa in the egg cytoplasm. This situation may also be due partly to the fact that a number of our insemination experiments were carried on at room temperature, when perhaps the normal fertilization reaction insuring monospermy is either inhibited or proceeds at a rate so slow that several sperm may enter before the reaction is completed.

Nonetheless, the recovery of normally cleaved ova indicates that ovarian eggs may be successfully fertilized even though they have not, at the time of the removal from the ovary, undergone the first maturation division. The fact that this occurs normally in the dog indicates that this is not as unlikely as it may seem, and implies that it does not occur normally in other mammals simply because the stimulus to maturation is attained in the follicle in these other species.

#### DISCUSSION

We believe that these experiments demonstrate clearly that the ovarian egg in the ripe follicles of mammals is activatable and fertilizable at any time. In another paper (Pincus and Enzmann) we have shown that once ovarian ova have reached full size and the nucleus has entered into the dictyate stage an isolation of the egg from the follicular epithelium frequently results in the formation of polar spindles with or without subsequent polar body formation, dependent apparently upon the rate of follicular atresia or the onset of ovulation. The data of this investigation show that the isolation of ova *in vitro* results in a similar sequence of nuclear changes. This implies that the associated follicle cells serve either to maintain the egg in a nutritional state wherein nuclear maturation is impossible, or that they actually supply to the ovum a substance or substances which directly inhibit nuclear maturation. It is notable that the preovulatory follicle characteristically contains few or no strands connecting the ovum to the follicular epithelium (*cf.* Pincus and Enzmann). It is interesting to note that in the dog (Evans and Cole, 1931) rupture of the follicular epithelium does not occur until ovulation (a complex folding goes on before ovulation, and the separation of the ovum from the epithelium presumably intervenes at a short interval before ovulation).

The obvious inference from these findings is that mammalian ovaries contain large numbers of fertilizable ova that never emerge from the ovary. If these ova can be obtained easily, one of the chief limitations to the direct study of mammalian eggs *in vitro*, namely the limited number of eggs ovulated, will be overcome. We have, in fact, obtained large numbers of eggs by puncturing follicles of various sizes. The uses to which these eggs have been put will be described in subsequent papers.

second polar body whereas a number of these ova with sperm do (see Table VI).

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|---------------------------|--------------------|-----------------|--|
| 0 hrs., from unmated does | Undiluted          | min. 20         | Sperm penetration takes place in most cases. Polyspermy is frequent. Male pronuclei may form. Polar bodies are not always present. The order of the process may become upset, for instance tetrads and a male pronucleus being present in the same egg |
| " "                       | "                  | hrs. 2          |  |
| " "                       | "                  | 4               |  |
| " "                       | "                  | 6               |  |
| " "                       | "                  | 7               |  |
| " "                       | "                  | 8               |  |
| " "                       | Diluted 1:4        | 16-24           | The same phenomena as in the upper series, but development seems to proceed further and with greater regularity. Second polar bodies are formed in many cases, especially in older eggs. The heavy sperm suspension leads to frequent polyspermy       |
| " "                       | " 1:10             |                 |  |
| 2 hrs. after mating       | Undiluted          | 26              |  |
| 4 " " "                   | "                  | 19              |  |
| 6 " " "                   | "                  | 20              |  |
| 7 " " "                   | "                  | 20              |  |
| 8 " " "                   | "                  | 20              |  |

sufficiently concentrated to insure sperm entry and not so dilute that the sperm will die before any entry is possible. Even with fairly dilute suspensions of active sperm we have found two or more spermatozoa in the egg cytoplasm. This situation may also be due partly to the fact that a number of our insemination experiments were carried on at room temperature, when perhaps the normal fertilization reaction insuring monospermy is either inhibited or proceeds at a rate so slow that several sperm may enter before the reaction is completed.

Nonetheless, the recovery of normally cleaved ova indicates that ovarian eggs may be successfully fertilized even though they have not, at the time of the removal from the ovary, undergone the first maturation division. The fact that this occurs normally in the dog indicates that this is not as unlikely as it may seem, and implies that it does not occur normally in other mammals simply because the stimulus to maturation is attained in the follicle in these other species.

#### DISCUSSION

We believe that these experiments demonstrate clearly that the ovarian egg in the ripe follicles of mammals is activatable and fertilizable at any time. In another paper (Pincus and Enzmann) we have shown that once ovarian ova have reached full size and the nucleus has entered into the dictyate stage an isolation of the egg from the follicular epithelium frequently results in the formation of polar spindles with or without subsequent polar body formation, dependent apparently upon the rate of follicular atresia or the onset of ovulation. The data of this investigation show that the isolation of ova *in vitro* results in a similar sequence of nuclear changes. This implies that the associated follicle cells serve either to maintain the egg in a nutritional state wherein nuclear maturation is impossible, or that they actually supply to the ovum a substance or substances which directly inhibit nuclear maturation. It is notable that the preovulatory follicle characteristically contains few or no strands connecting the ovum to the follicular epithelium (cf. Pincus and Enzmann). It is interesting to note that in the dog (Evans and Cole, 1931) rupture of the follicular epithelium does not occur until ovulation (a complex folding goes on before ovulation, and the separation of the ovum from the epithelium presumably intervenes at a short interval before ovulation).

The obvious inference from these findings is that mammalian ovaries contain large numbers of fertilizable ova that never emerge from the ovary. If these ova can be obtained easily, one of the chief limitations to the direct study of mammalian eggs *in vitro*, namely the limited number of eggs ovulated, will be overcome. We have, in fact, obtained large numbers of eggs by puncturing follicles of various sizes. The uses to which these eggs have been put will be described in subsequent papers.

We should, finally, consider one possible objection to the findings here reported. It may be declared that the maturation figures of our cultured ova are the results of atresia and not of activation. If that were the case then normal maturation would be also a process of atresia, since the events occurring *in vivo* and *in vitro* are indistinguishable morphologically and in the rate of their progress. True atresia may be said to occur only when an ovum is no longer fertilizable (for further discussion see Pincus, 1936).

## SUMMARY

1. A definite chronological sequence of events occurs in the eggs and follicles of rabbits after mating or after the injection of ovulation-inducing substances. The follicle secretes secondary liquor folliculi, and there occurs a separation of the corona radiata from strands connecting it to the follicle cells. The ovum goes through nuclear maturation with as climax the production of the first polar body by the 8th hour after copulation.

2. Thyroxin injections cause indirectly the same effects as mating or pituitary injections but no ovulation occurs. The thyroxin effect occurs later than the pituitary effect and is due to an initiation of atresia in the follicles.

3. Explantation of ova results in typical maturation phenomena which are apparently unaffected by the presence of pituitary hormones or of thyroxin in the culture medium.

4. It is concluded that maturation of the ovum can be obtained simply by isolating it from the normal follicular environment.

5. Normal fertilization can be secured with eggs removed from the follicles.

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## EXPLANATION OF PLATES 29 AND 30

FIG. 1. Ovarian egg obtained by puncture of a follicle from the ovary of an unmated rabbit.

FIG. 2. Ovarian egg obtained by puncture of a follicle from the ovary of a doe mated 2 hours previously. The chromatin material condenses to tetrads. The vesicular membrane is still present.

FIG. 3. Ovarian egg from a doe mated 4 hours previously. Tetrads fully formed and vesicular nucleus dissolved.

FIG. 4. Ovarian egg from a doe mated 5 hours previously. The tetrads have become smaller and arranged themselves in a plate. All traces of the vesicular membrane have disappeared.

FIG. 5. Ovarian egg from a doe mated 6 hours previously. The first polar spindle begins to form.

FIG. 6. Ovarian egg from a doe mated 8 hours previously. The first polar body has been given off.

FIG. 7. Ovarian egg from a doe mated 9 hours previously. First polar body and second polar spindle.

FIG. 8. Ovarian egg cultured for 24 hours in Ringer-Locke solution containing maturity hormone. Note apparent fusion nuclei.

FIG. 9. Ovarian egg from a doe which had received 2 cc. thyroxin intravenously. Tetrads have formed in a vesicular nucleus.

FIG. 10. Ovarian egg from unmated doe, inseminated with normal sperm *in vitro*. Sperm penetration has occurred. Note sperm head at lower right periphery.

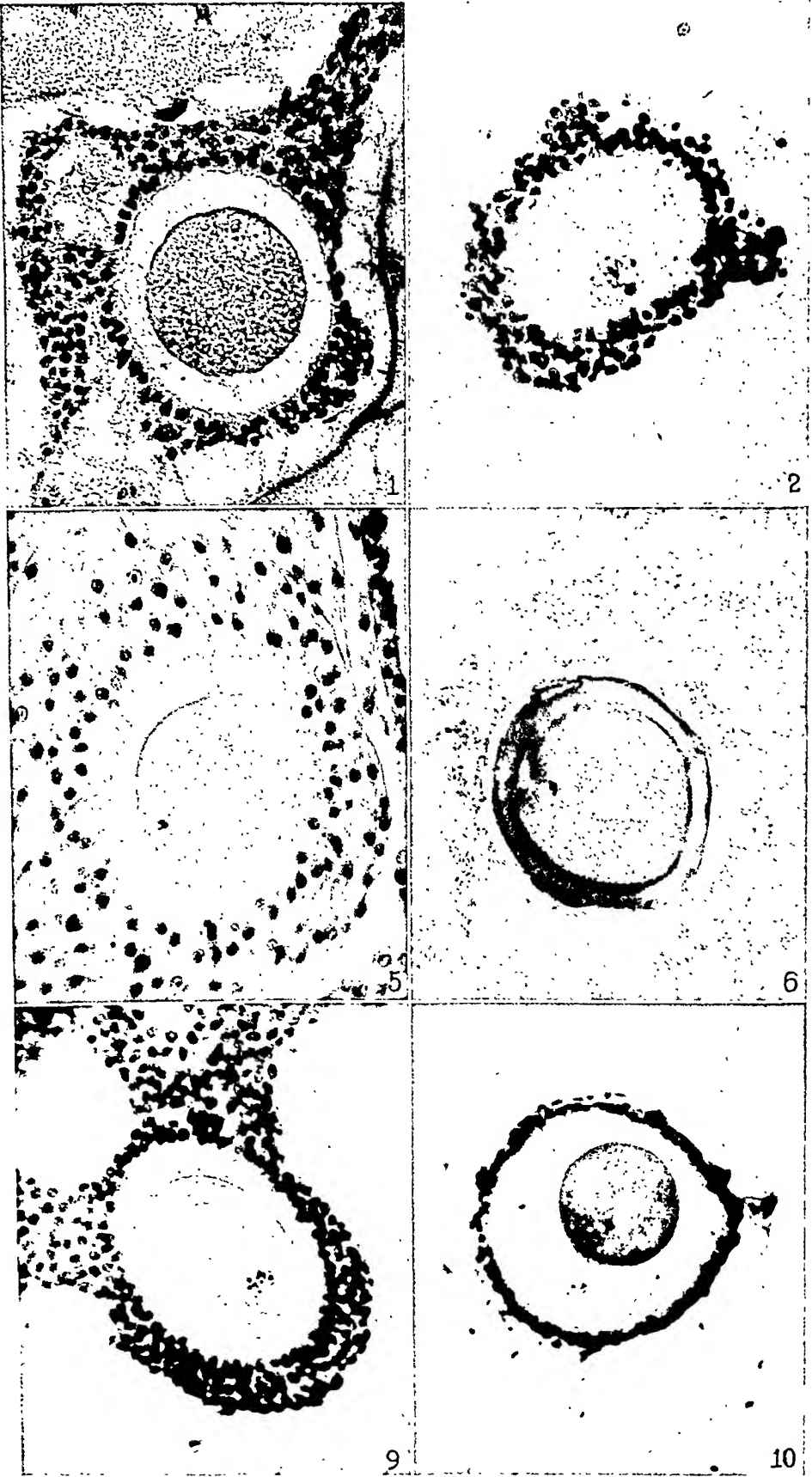
FIG. 11. Ovarian egg from a doe mated 6 hours previously and inseminated *in vitro* with normal sperm. Male and female pronuclei present side by side.

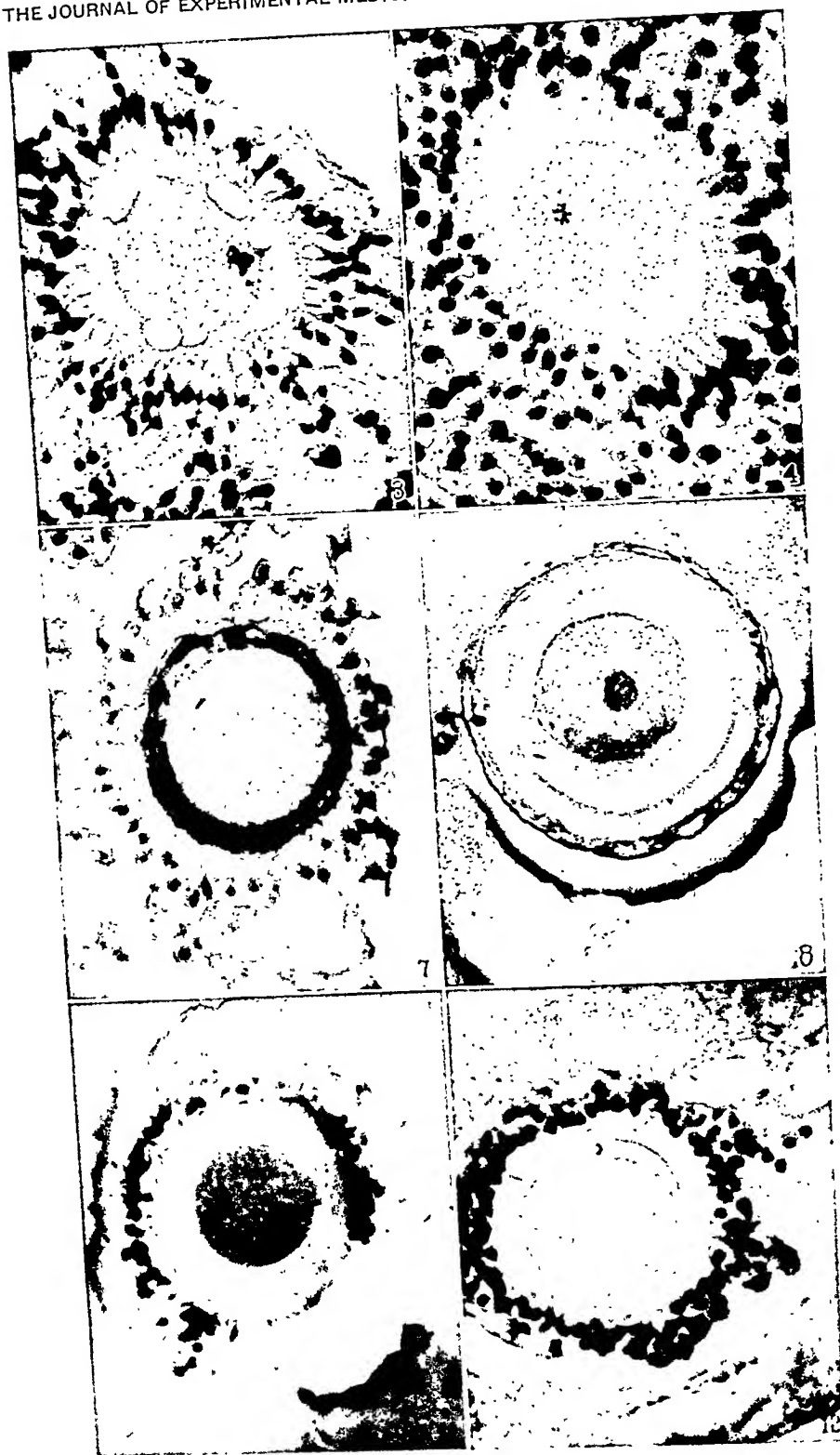
FIG. 12. Ovarian egg from a doe mated 8 hours previously and inseminated with normal sperm. The first polar body has formed and sperm penetration occurred. The entering spermatozoan has formed a male pronucleus (center left).













# EPIDEMIOLOGY OF EQUINE ENCEPHALOMYELITIS IN THE EASTERN UNITED STATES

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Although it has probably existed for many years in the United States, equine encephalomyelitis was not recognized as a separate entity until 1930 when Meyer, Haring, and Howitt (1) demonstrated a filtrable virus as the cause of the condition. Since then the disease has been found in various western states, and in the summer of 1933 a similar condition was recognized in Virginia, Delaware, New Jersey, and Maryland. Although the equine disease was much the same as that described in the West and the virus isolated was pathogenic for the same species of animals, it differed serologically (2) from the virus obtained by Meyer. We have therefore referred to the disease with which we have been working as the eastern type of encephalomyelitis, and it is our purpose here to record various facts that may have a bearing on its transmission as a background for experiments that are to follow.

## *Seasonal Distribution*

Like the western disease the one found in the East has a seasonal distribution; in the last two years it has appeared in August, reached its height during September, and disappeared in October. We have had reports of winter cases but the diagnoses were clinical ones and in the few instances where we were able to get brain material for examination there has been no evidence of the virus disease either histologically or by animal inoculation. Forage poisoning (3) and leucoencephalitis (4) are often confused with the virus disease and may occur in the same regions. A positive diagnosis of equine encephalomyelitis in winter cases should therefore be substantiated by more than the clinical picture, preferably by the demonstration of

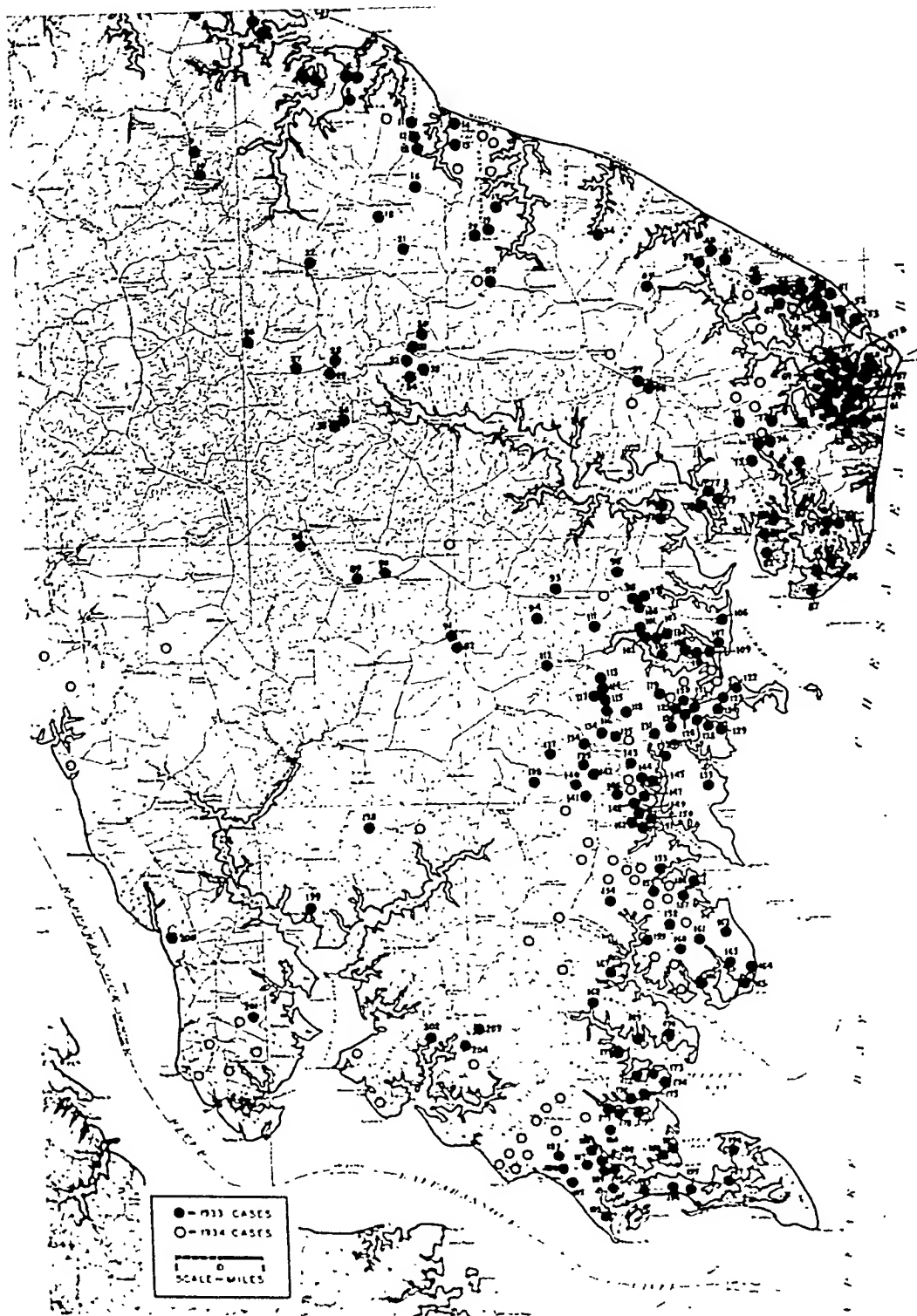
the virus. In the region to be described, where we have the cooperation of a very intelligent county agent, winter cases have not been detected although up till the time of frost many cases were found.

### *Geographic Distribution*

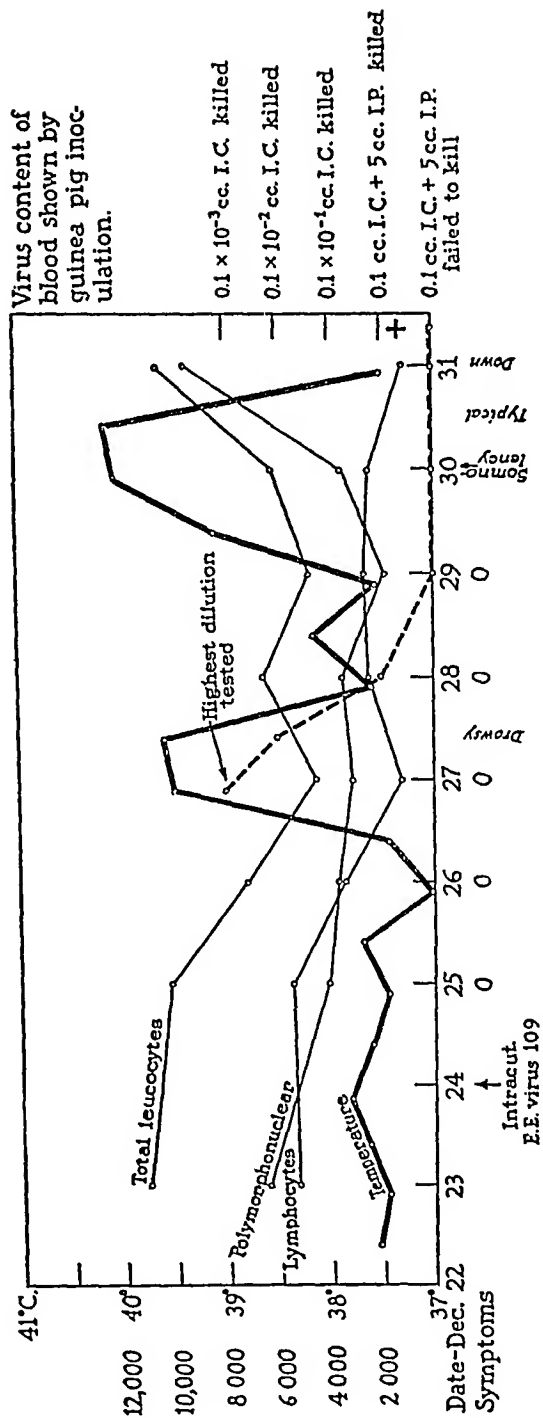
Unlike the western disease the cases that we have encountered have been closely related to salt marsh areas. This is brought out in Text-fig. 1 which shows the distribution of infected farms on the Northern Neck of Virginia during the years 1933 and 1934. These data were secured for us by Dr. H. C. Givens, State Veterinarian, and by Mr. C. Carter Chase. It will be noted that the great majority of cases are within 2 miles of the coast line which has many inlets and much marshy ground. There were cases of the disease in the inland area where the horse population is greater than along the shore, but in several instances they were in horses that had been carting produce to the shore. In other instances no such history was obtained, so that we can say that the disease is not strictly limited to salt marsh areas. While we have been unable to secure data which would enable us to make similar maps for other regions, the information we have obtained from the Eastern Shore of Virginia, from Delaware, and from southern New Jersey shows that the disease incidence is far greater near the coast line than it is inland. In the summer of 1934 more cases were detected inland in New Jersey, but the great majority were in horses used in gathering hay from the salt marshes. Since the disease is not reportable figures on the distribution are only approximate.

### *Consideration of Contact Infection*

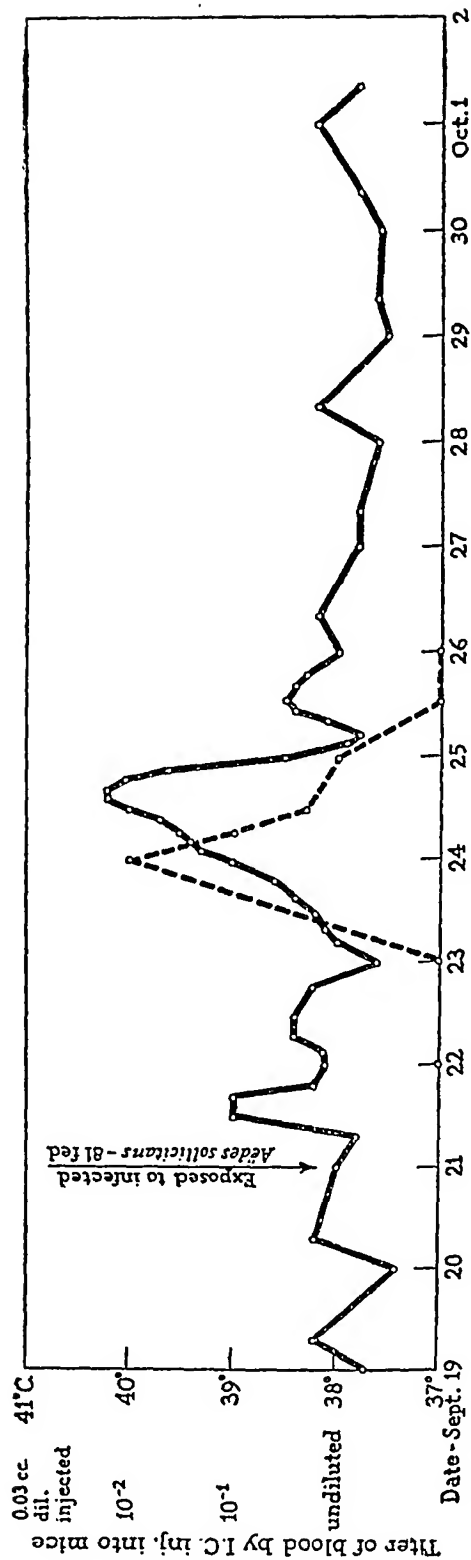
Influenced by the work that has been done on the transmission of poliomyelitis our first thought was that equine encephalomyelitis was also spread by contact. The more conditions in the field were observed, the less likely it seemed that the two diseases were transmitted in the same way. As noted in the preceding paragraph, the cases were sharply localized to regions near the shore and there was very little tendency for it to spread inland. On some farms all horses developed the disease but on many others only a portion were infected. Often the disease appeared on farms a mile or more distant from those on which cases had occurred and there would be no known



TEXT FIG. 1



TEXT-FIG. 2. Horse 194. I. C. = intracerebral injection. I. P. = intraperitoneal injection.



TEXT-FIG. 3. Horse 249.

characteristic symptoms of the disease. Moreover this diphasic temperature with virus in the blood stream during the first rise is found regularly in guinea pigs that are inoculated subcutaneously. It appears to us to be a regular feature of the disease.

In Text-fig. 3 is a chart of another horse which gives us some information about transmission. We shall not discuss the inoculation of this animal as it will be referred to in a subsequent paper. Two days after exposure the temperature began to rise and, as will be seen, at the same time the virus appeared in the blood stream. When the temperature came down virus was no longer demonstrable. There was only this one temperature rise and the animal showed absolutely no central nervous system or other symptoms. Whereas the blood before the exposure contained no virus-neutralizing antibodies, after the temperature rise they were demonstrated to be present. Furthermore some 5 weeks after the temperature rise the animal was inoculated intracerebrally with virus. It showed no temperature rise and no symptoms of the disease and was subsequently disposed of. A control animal inoculated at the same time with the same material developed encephalomyelitis and died. This, then, is an abortive case of the disease and we believe that such are not uncommon, for, as stated earlier, sera from animals that are believed never to have been sick but kept in districts where the disease has occurred, frequently show immune bodies. Guinea pigs inoculated subcutaneously with virus often develop only one temperature rise and show no central nervous system symptoms. When after 2 to 3 weeks they are tested for immunity by the intracerebral route they are found to resist the virus whereas control animals come down. It is obvious that abortive cases in horses are just as much a source of virus for biting insects as animals showing symptoms.

#### *Possible Reservoir Host*

The virus curve shown in Text-fig. 2 may not be typical of the natural infection but it probably closely approximates it. It will be seen that the amount of virus falls off rapidly and that the time when biting insects could infect themselves is a relatively short one. If the horse is the only source of virus the transmitters must feed frequently or be present in enormous numbers.



It is possible, however, that the horse is a secondary host, to which the virus is transmitted from another species. Little is known about reservoir hosts in virus diseases but they may be of great importance. The wart hog carries a hog cholera-like virus (9) that it transmits to domestic swine, and Shope (10) has good evidence that pseudorabies is a very mild but contagious disease of swine which is rarely recognized, and that the highly fatal disease in cattle is contracted from infected pigs.

Various animals are susceptible to equine encephalomyelitis virus when it is injected intracerebrally, and rabbits, guinea pigs, and mice can be infected by subcutaneous inoculation. Giltner (11) found the pigeon susceptible to intracerebral inoculation and suggested that it might be involved in the transmission of the disease. We know practically nothing about the susceptibility of the great variety of wild animals and birds that are so closely associated with our domestic animals.

If there is a reservoir host it must be one that covers a large amount of territory, for the disease appears at approximately the same time in areas that are separated from one another by barriers that can be traversed by only a few forms. For example, the Northern Neck of Virginia and the Eastern Shore are separated by the Chesapeake Bay which is from 20 to 25 miles wide, yet the disease has appeared in these two regions at about the same time the past 2 years.

Man is the most widely traveled mammal and must be considered as a possible reservoir host. Meyer (12) suggested that man was susceptible to the virus, for he learned of three human cases of encephalitis that had been in contact with infected horses. No virus was secured from these cases and no tests for neutralizing antibodies were made on the sera from the two cases that recovered. We have made inquiries from doctors practicing in the regions where the disease was prevalent and have been unable to get a history of any human infections that resembled encephalitis. Our experience in the laboratory leads us to believe that man is not very susceptible to the virus. Six of us have been working with the disease for over 2 years, and although precautions have been taken, accidental contacts with infected material have frequently occurred; yet not one of us has developed a disease resembling encephalomyelitis, and the sera of all are free from neutralizing antibodies.

From the above it seems hardly probable that man is a reservoir host for the virus. Birds should be considered as possible hosts and are made suspect by the epidemiological findings. We have, however, no facts that support such an hypothesis and will not consider it further at the present time.

#### SUMMARY

Equine encephalomyelitis of the eastern type is a disease of the late summer and fall and cases are found in greatest numbers near salt marshes. The epidemiological findings are against its transmission by contact and favor the view that it is insect borne. Although virus can be demonstrated in the blood of infected horses it is present for a relatively short time, and the possibility that the disease is not primarily an infection of horses but that it is transmitted to them from another host is considered.

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# THE TRANSMISSION OF EQUINE ENCEPHALOMYELITIS VIRUS BY *AËDES AEGYPTI*

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Kelser's demonstration (1) that *Aëdes aegypti* will transmit the virus of equine encephalomyelitis from infected to normal guinea pigs provides an almost ideal combination for the study of insect transmission of this type of infection. The mosquitoes can be raised with ease the year round, and guinea pigs are highly susceptible to the virus, which produces in them a characteristic disease. It is our purpose to record here certain observations that followed attempts to confirm Kelser's work.

## Methods

To Dr. A. Watson Sellards we are indebted for the original stock of *Aëdes aegypti* eggs as well as directions which enabled us, with the assistance of Dr. Lacaille, to establish a colony quickly. For transmission experiments females that had recently emerged and that had never been given a blood meal were transferred to small screened cages. These mosquitoes could be infected by feeding either on a guinea pig inoculated with encephalomyelitis virus or on a mixture of the brain from an infected guinea pig and defibrinated horse blood. When mosquitoes are to be fed on a living animal, their food, consisting of 3 per cent sugar solution, is withheld for 3 days. They will then readily attack a guinea pig that is immobilized and placed in their cage. The infection of mosquitoes by feeding on a mixture of virus and blood requires a more careful preparation of the insects. For 4 days they are allowed only water and this is withheld the night before they are fed. The mixture then offered them consists of a 10 per cent suspension of brain from an infected guinea pig in saline and an equal part of defibrinated horse blood. A pledget of cotton in each of two small Petri dishes is moistened with the mixture and a little powdered sugar is sprinkled over the surface. One dish is placed in the refrigerator and the other in the cage. The hungry mosquitoes feed on the mixture, which is allowed to remain in the cage for an hour and is then replaced by the one that has been held in the refrigerator, since it is known that the virus deteriorates rapidly at room temperature. By withholding water for 1 day and sugar solution for 2 days the mosquitoes that have

not fed die of starvation. After this time they are fed daily a 3 to 5 per cent sugar solution which is poured over cotton placed on the top of their screen wire cage. A piece of cotton moistened with water is also kept on the cage. As will be seen from the tables, mosquitoes have been kept alive for long periods of time on this diet.

### *Infection of Mosquitoes with Western Virus*

Using the same strain of western virus our first attempts to confirm Kelser's work were negative. However, a successful transmission from an infected to a normal guinea pig was finally secured and it was noted that in this case the virus content of the blood of the infected animal was unusually high. Following this observation several lots of mosquitoes were fed on a guinea pig at intervals after inoculation, and at the time of feeding blood was withdrawn and titrated for its virus content. The mosquitoes were later tested for their ability to transmit the virus to normal guinea pigs and it was found that only those lots that fed on the inoculated animal when the test showed that the virus content of the blood was high were later able to transmit the disease. Since the success or failure of transmission experiments may depend on this point further experiments were made, one of which follows.

Five hundred normal female *Aedes aegypti* were divided into four equal lots and each lot was fed on defibrinated horse blood mixed with different dilutions of brain from an infected guinea pig. The virus content of this brain was such that disease was produced in a guinea pig by the injection into the foot pads of  $1 \times 10^{-6}$  cc. of the 10 per cent suspension of the guinea pig brain. In this experiment the dilutions were made with a 10 per cent suspension of normal guinea pig brain in salt solution, but in other experiments salt solution alone was used as a diluent and the same results obtained. The results of the experiment are given in Table I.

Immediately after feeding, virus was demonstrated in the mosquitoes withdrawn from the lot fed on the highest dilution of infected brain, so that it must have been present in all lots. When tested for their ability to transmit at different time intervals, only the mosquitoes that received the undiluted and the 1 per cent brain suspension were able to infect guinea pigs. Furthermore at 28 days, which ended the experiment, virus could be demonstrated by inoculation only in these first two lots. The experiment, the results of which

coincide with others like it, shows that to secure positive transmission by *Aedes aegypti* the mosquitoes must be fed virus of high concentration. When fed lower concentrations the virus is soon lost and transmission experiments are negative.

Experience has shown us that in order to infect the mosquitoes from guinea pigs inoculated with the virus the feeding must take place within a relatively narrow period of time. The practice we have finally adopted is to give the animals an intracerebral injection

TABLE I

*Amount of Western Virus Necessary to Infect Aedes aegypti. Brain Virus Diluted with 10 Per Cent Suspension of Normal Guinea Pig Brain and Mixed with an Equal Amount of Defibrinated Horse Blood*

| Fed 10 per cent suspension of virus diluted | Virus shown by guinea pig pad inoculation of 1 cc. suspension of 10 mosquitoes in 2.5 cc. saline |                       | Transmission to guinea pigs by feeding |        |          |        |          |        |          |        |
|---|--|-----------------------|--|--------|----------|--------|----------|--------|----------|--------|
|   | Immediately after feeding  | 28 days after feeding | 7th day                                |        | 14th day |        | 21st day |        | 28th day |        |
|   |  |                       | No. fed                                | Result | No. fed  | Result | No. fed  | Result | No. fed  | Result |
| Undiluted                                   |  | +                     | 20                                     | +      | 24       | +      | 25       | +      | 24       | +      |
| 1:10  |  | +                     | 20                                     | +      | 21       | +      | 25       | +      | 25       | +      |
| 1:100                                       |  | 0                     | 20                                     | 0      | 70       | 0      | 80       | 0      | 65       | 0      |
| 1:1000                                      | +  | 0                     | 20                                     | 0      | 65       | 0      | 75       | 0      | 75       | 0      |

\* In this and all the remaining tables, + indicates that the guinea pigs showed a typical temperature, symptoms and death, or temperature and subsequent immunity. 0 indicates no temperature and no immunity.

of 0.1 cc. of the 10 per cent suspension of infected brain and in addition 0.25 cc. into the pad of each hind foot. The mosquitoes are fed as soon as possible after the temperature of the animals reaches 40.5°, which is from 18 to 30 hours after the inoculation. If fed at this time mosquitoes usually transmit the disease when tested subsequently, but if the feeding is delayed the transmission experiments are either negative or irregular.

In Table II are the results of an experiment made to determine the interval between feeding and the time when *Aedes aegypti* would

## TRANSMISSION OF EQUINE ENCEPHALOMYELITIS

United States. Our attempts to infect *Aedes aegypti* with this eastern strain of virus by allowing them to feed on infected brain of high titer or on infected guinea pigs have been essentially negative. In Tables III and IV are examples of such experiments. In the latter table,

TABLE III

*Ability of Aedes aegypti to Retain and Transmit Eastern and Western Strains of Equine Encephalomyelitis Virus to Guinea Pigs after Feeding on Brain Virus + Horse Blood*

| Days after feeding on virus | Western virus          |                         |        | Eastern virus          |                         |        |
|-----------------------------|------------------------|-------------------------|--------|------------------------|-------------------------|--------|
|                             | Virus in 10 mosquitoes | Transmission by feeding |        | Virus in 10 mosquitoes | Transmission by feeding |        |
|                             |                        | No. fed                 | Result |                        | No. fed                 | Result |
| Immediately                 | +                      |                         |        | +                      |                         |        |
| 3                           | +                      | 50                      | 0      | +                      | 45                      | 0      |
| 7                           | +                      | 32                      | +      | 0                      | 25                      | 0      |
| 14                          | +                      | 21                      | +      | 0                      | 18                      | 0      |

TABLE IV

*Ability of Aedes aegypti to Transmit Eastern and Western Strains of Equine Encephalomyelitis Virus from Infected to Normal Guinea Pigs*

| Days after feeding on infected guinea pig | Western virus |        | Eastern virus |        |
|---|---------------|--------|---------------|--------|
|   | No. feeding   | Result | No. feeding   | Result |
|   |               |        |               | 0      |
| 4   | 28            | +      | 25            | +      |
| 5   | 16            | +      | 14            | 0      |
| 7   | 25            | +      | 25            | 0      |
| 11  | 25            | +      | 25            | 0      |
| 20  | 35            | +      | 18            | 0      |
| 30  | 40            | +      |               |        |

The titration of a suspension of 10 mosquitoes from each lot in salt solution immediately after the infecting meal showed that both had approximately the same amount of virus.

the mosquitoes fed on a guinea pig infected with eastern virus transmitted the disease on the 5th day but subsequent feedings were negative. This is the only positive transmission that we have obtained of the eastern virus.

Storey (4) has reported some interesting experiments with a strain of leafhoppers that was incapable of transmitting the streak virus of corn. He found that he could inoculate them by puncturing the abdomen with an infected needle or by allowing them to feed on a diseased plant and then puncturing with a clean needle. After either method of inoculation they were capable of transmitting the virus

TABLE V  
*Effect of Puncturing the Abdomen after an Infective Blood Meal on the Ability of Aedes aegypti to Transmit Equine Encephalomyelitis Virus of the Eastern Type*

| Days after<br>infective meal<br>when mosqui-<br>toes fed on<br>normal guinea<br>pigs | Experiment 1 |        |            |        | Experiment 2 |        |            |        | Experiment 3 |        |            |        |
|--|--------------|--------|------------|--------|--------------|--------|------------|--------|--------------|--------|------------|--------|
|  | Punctured    |        | Control    |        | Punctured    |        | Control    |        | Punctured    |        | Control    |        |
|  | No.<br>fed   | Result | No.<br>fed | Result | No.<br>fed   | Result | No.<br>fed | Result | No.<br>fed   | Result | No.<br>fed | Result |
|  |              |        |            |        |              |        |            |        |              |        |            |        |
| 7  | 46           | +      | 45         | 0      | 55           | +      | 35         | 0      | 35           | +      | 50         | 0      |
| 14   | 45           | +      | 63         | 0      | 24           | +      | 46         | 0      | 33           | +      | 50         | 0      |
| 21   | 35           | +      | 30         | 0      | 18           | +      |            |        |              |        |            |        |
| 28   | 30           | +      | 50         | 0      | 16           | 0      |            |        | 33           | +      | 50         | 0      |
| 35   | 38           | +      | 40         | 0      | 45           | +      | 36         | 0      | 40           | +      | 55         | 0      |
| 42   | 35           | 0      | 42         | 0      | 23           | 0      | 38         | 0      | 35           | 0      | 52         | 0      |
| 49   | 35           | 0      | 53         | 0      | 20           | +      |            |        |              |        |            |        |
| 56   | 30           | +      | 40         | 0      | 31           | +      | 35         | 0      | 33           | +      | 33         | 0      |
| 63   | 26           | ++     | 44         | 0      | 25           | +      | 30         | 0      | 23           | 0      | 50         | 0      |
|  |              |        |            |        | 17           | +      | 25         | 0      | 5            | 0      | 26         | 0      |
|  |              |        |            |        | 11           | +      | 23         | 0      | 0†           | 0      | 10         | 0      |
|  |              |        |            |        | 10           | ++     | 23         | 0      | 15           | 0‡     | 26         | 0      |

\* A suspension of 16 remaining mosquitoes 90 days after the initial feeding produced encephalomyelitis in a guinea pig.

† A suspension of 6 remaining mosquitoes 79 days after the initial feeding produced encephalomyelitis in a guinea pig.

‡ A suspension of 6 remaining mosquitoes 71 days after the initial feeding failed to produce encephalomyelitis in a guinea pig.

to normal plants. We have repeated this type of experiment with *Aedes aegypti* and the eastern virus. Table V contains the results of three separate experiments in which the mosquitoes were allowed to feed on infected guinea pigs and half of them were then punctured with a small sewing needle. When the punctured mosquitoes were allowed to feed on normal guinea pigs infection resulted, whereas the control mosquitoes that had fed on the guinea pigs at the same time



## TRANSMISSION OF EQUINE ENCEPHALOMYELITIS

but had not been punctured invariably failed to transmit the disease. However, if the punctured mosquitoes were retained for approximately 6 weeks infection did not always follow their feeding.

Attempts were also made to inoculate mosquitoes by piercing their abdomens with a small needle that had been moistened in virus suspensions. If mosquitoes with flat abdomens are punctured the mortality is about 90 per cent, but when their abdomens are distended the puncture is less harmful. In Table VI are the results of two ex-

TABLE VI

*Ability of Aedes aegypti to Transmit Equine Encephalomyelitis Virus of the Eastern Type after Their Abdomens Had Been Punctured with a Needle Moistened in Virus Suspension*

| Days after inoculation when mosquitoes fed on normal guinea pigs | Experiment A |        | Experiment B |        |
|--|--------------|--------|--------------|--------|
|  | No. feeding  | Result | No. feeding  | Result |
| 8  | 25           | +      | 40           | +      |
| 15   | 23           | +      | 32           | +      |
| 22   | 15           | +      | 22           | +      |
| 29   | 20           | +      | 25           | 0      |
| 35   | 15           | +      | 30           | 0      |
| 43   | 15           | 0      | 27           | 0      |
| 50   | 12           | 0      | 20           | 0      |
| 57   | 15           | 0      | 21           | 0      |
| 63   | 12*          | 0      | 20†          | +      |

\* A suspension of 9 remaining mosquitoes 68 days after the initial feeding produced encephalomyelitis in a guinea pig.

† A suspension of 17 remaining mosquitoes 68 days after the initial feeding produced encephalomyelitis in a guinea pig.

periments. The Lot A mosquitoes were allowed to feed on normal horse blood and their abdomens then punctured with the needle moistened with virus, while Lot B were fed on sugar solution previous to the puncture. In Experiment A transmission was obtained up to the 35th day, whereas in Experiment B there was no transmission after the 22nd day until the 63rd day. In both experiments virus was demonstrated on the 68th day after inoculation by the injection of suspensions of mosquitoes from each lot into normal guinea pigs. The inoculation of mosquitoes with a virus to which they are

ordinarily resistant suggests that under certain conditions a similar process may occur in nature. For example, if mosquitoes of a certain region had an intestinal infection, virus taken in at a blood meal might penetrate the mucosa and be transmitted at the next feeding. If this occurred repeatedly the virus might become adapted to the mosquitoes and be able to infect normal individuals. Thus a new transmitter of a disease might conceivably arise.

#### SUMMARY

In confirming Kelser's work on the transmission of equine encephalomyelitis of the western type by *Aedes aegypti* it has been learned that the mosquitoes must be fed virus of high titer if positive results are to be secured. A period of from 4 to 5 days after feeding either on infected guinea pigs or on brain containing virus must elapse before the disease is transmitted by biting, but after this time transmission regularly results for a period of about 2 months. By inoculation, virus can be demonstrated in the bodies of infected mosquitoes for the duration of life.

Although virus multiplies in the mosquitoes and is generally distributed in their bodies, repeated attempts to demonstrate it in the eggs from females known to be infected as well as in larvae, pupae, and adults from such eggs have been uniformly negative. Larvae have not taken up virus added to the water in which they were living. Male mosquitoes have been infected with virus by feeding but they have not transmitted the virus to normal females, nor have males transmitted the virus from infected to normal females.

When virus of the eastern instead of the western type is used transmission experiments with *Aedes aegypti* are negative. Apparently this virus is incapable of penetrating the intestinal mucosa of the mosquito. If, however, it is inoculated into the body cavity by needle puncture it persists and transmission experiments are positive.

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# A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

## III. THE REACTION BETWEEN CRYSTALLINE EGG ALBUMIN AND ITS HOMOLOGOUS ANTIBODY\*

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In the first paper of this series (1)<sup>1</sup> it was shown that the precipitin reaction might be considered the resultant of a series of competing bimolecular reactions, the quantitative outcome of which depended on the relative proportions in which the components were mixed. It was thus found possible to express the entire course of the precipitin reaction between a specific polysaccharide and its homologous antibody by simple equations derived from the mass law. In the second paper of the series (2) it was shown that these considerations were equally applicable to an antigen-antibody system in which the antigen was R-salt-azo-biphenyl-azo-crystalline egg albumin. This antigen, a deep red dye, could be determined with accuracy in precipitates over the entire range of the reaction, thus permitting the separate quantitative estimation of the amounts of antigen and antibody nitrogen precipitated.

In the present communication the information obtained with the aid of the specific polysaccharide and the protein dye is applied to a system involving a colorless antigen, crystalline egg albumin, and its homologous antibody. The antigen used has the advantages of homogeneity, known molecular weight, and of having been studied quantitatively in respect to its behavior in the precipitin reaction by a number

\* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

<sup>1</sup> A statement was omitted in this paper that the sera used were absorbed with pneumococcus protein and "C" substance before purification of the antibody.

of workers including Taylor (3), Culbertson (4, 5), Taylor, Adair, and Adair (6), and Hooker and Boyd (7). It is shown below that the theory presented in Papers I and II of this series is applicable to the egg albumin-antibody system and that the data of the previous workers with this reaction may be used over the portion of the range in which they are sufficiently explicit. A method is given by which both egg albumin and antibody nitrogen may be determined quantitatively in precipitates and supernatants over a limited range in the region of antigen excess. Except in the region approaching complete inhibition it is thus found possible to predict the behavior of an anti-egg albumin serum over the entire remaining range of the reaction after a small number of quantitative analyses for nitrogen have been made. Moreover, the empirical relation which was shown in Reference 2 to permit the calculation of the maximum amount of specifically precipitable antibody has been found to be applicable, though somewhat less exactly, to the egg albumin-antibody system.

Opportunity is also taken to discuss the concept of the equivalence zone, the constancy of the equivalence point, the relation of the findings to methods for the determination of optimal proportions for flocculation, and certain practical considerations regarding the combining proportions of antigen and antibody.

Finally, a quantitative study has been made of the behavior of antibody formed by a single rabbit in three successive courses of injections. The differences found are believed to have bearing on the mechanism of antibody formation and the process of immunization.

#### EXPERIMENTAL

The writers are pleased to note that in all of the four laboratories studying the egg albumin-antibody reaction the amount of specifically precipitable nitrogen is now accepted as a standard. The technique used in this laboratory for analyzing specific precipitates for nitrogen has been given in previous papers (1, 2, 8, 9). In applying the method to the egg albumin (Ea)-antibody (A) system it was found that precipitates, particularly in the equivalence zone, tended to remain somewhat loosely packed, so that it was necessary in such cases to centrifuge the supernatant a second time to recover additional small amounts of specific precipitate. In such cases tubes must be carefully watched if allowed to drain.

The crystalline egg albumin used was prepared according to Reference 10 and was recrystallized three times and dialyzed in the cold until free from ammonium salts. The nitrogen content of Ea was taken as 15.5 per cent.

TABLE I

*Influence of Temperature, Time of Standing, and Volume on Total Specifically Precipitable Nitrogen*

| Laboratory No. of serum<br>+ amount Ea N | Total N precipitated from 1.0 ml. serum by amount of Ea N indicated in<br>1.0 ml. saline.—Total volume 2.0 ml. unless otherwise stated |                |                |                           |                |                               |
|--|--|----------------|----------------|---------------------------|----------------|-------------------------------|
|  | 37°,<br>1 hr.  | 37°,<br>2 hrs. | 37°,<br>3 hrs. | 20°, 0°*                  | 0°,<br>24 hrs. | 0°, 48 hrs.                   |
|  | mg.  | mg.            | mg.            | mg.                       | mg.            | mg.                           |
| 1.31<br>+0.027 mg. Ea N                  | 0.29   | 0.29           | 0.29           |                           |                | 0.31                          |
| 2.24<br>+1.0 mg. Ea N                    |  |                |                | 1.78, 1.74<br>1.75, 1.72† |                |                               |
| 3.87 I, 1:1<br>+ 0.050 mg. Ea N          | 0.62   | 0.61           |                |                           |                | 0.63                          |
| + 0.098 mg. Ea N<br>(excess)             | 0.82   | 0.81           |                |                           | 0.82           | 0.82                          |
|  |  |                |                | 37°, 0°                   |                |                               |
| + 0.124 mg. Ea N                         |  |                |                | 0.72<br>0.70 (6 ml.)      |                |                               |
|  |  |                |                | 20°, 0°                   |                |                               |
| + 0.305 " " "                            |  | 0.19           |                | 0.12                      |                | 0.11‡                         |
| 3.87 II, 1:1<br>+ 0.050 mg. Ea N         |  |                |                |                           |                | 0.84, 0.85                    |
| + 0.098 " " "                            |  |                |                |                           |                | 0.82§<br>1.18<br>1.15 (9 ml.) |
| 3.87 III, 1:1<br>+0.079 mg. Ea N         |  |                |                |                           |                | 1.31  <br>1.28 (8 ml.)        |
| 3.88<br>+ 0.066 mg. Ea N                 |  | 0.70           |                |                           |                | 0.75                          |

\* 2 hrs. at 20°, overnight in the ice box.

† 4 ml. volume.

‡ Value given by duplicate tubes standing 4 days and 7 days in the ice box, with occasional stirring.

§ Two additional washings with a total of approximately 4 ml. chilled saline.

|| Nitrogen determined according to Reference 11.

In Table I are given data showing the effect of temperature and volume changes on the amount of nitrogen precipitated. Since occasional anti-Ea sera gave 0.01 or 0.02 mg. more specifically precipitable N per ml. when allowed to stand at 0° for 48 instead of 24 hours, all determinations at 0° were allowed to stand 2 days except those in the inhibition zone, for which 4 days were allowed (*cf.* 2).

Egg albumin suspensions were prepared for injection as follows: Dialyzed solutions of crystalline Ea were diluted with saline to less than 1 per cent Ea and were treated with 1 ml. of sterile 1 per cent alum solution per 100 mg. Ea present. The clear solution was neutralized with N sodium hydroxide solution until the resulting precipitate no longer appeared to increase, the maximum being reached when the suspension was barely acid to litmus paper. The suspension, which contained very little dissolved Ea, was brought down to the desired concentration (5 mg. Ea per ml. in the case of Rabbit 3.87) with saline and enough 1 per cent merthiolate solution to make a final concentration of 1:10,000. Rabbits were injected with this suspension four times a week for 4 successive weeks (Course I) and generally yielded excellent antisera. Rabbit 3.87 (weight 4.9 kilos) received fourteen injections of 10 mg. each under this schedule, and the serum designated 3.87 I was obtained 5 days after the last injection. Serum 3.87 II was secured 5 days after completion of a further course of eleven injections starting with 2.5 mg. Ea and working up to 15 mg. at the end, and 3.87 III after six more injections following a rest period of over a month.<sup>2</sup> The serum then contained 85 mg. of total protein per ml. (total N  $\times$  6.25), of which 44.7 mg. per ml. consisted of globulin (Howe method). It will be noted that by this time 21.5 per cent of the total protein, or 40.7 per cent of the globulin, consisted of precipitin (maximum specifically precipitable N  $\times$  6.25). Quantitative data on the sera from the three courses are given in Table II and compared in graphic form at a concentration of 1 mg. antibody N per ml. in Text-fig. 2, while Text-fig. 1 gives the total N and antibody N curves of 3.87 I as well as the data on this serum calculated according to equations [3] and [6]. In Table II and other Tables, N estimations (always in duplicate unless otherwise stated) are reported to the third decimal place although its value is uncertain except in the case of Ea N added.

The analysis of supernatants for the very small amounts of Ea in the limited range of Ea excess before the beginning of the inhibition zone is most simply carried out by adding as large an aliquot as possible to the same amount of serum as was used in the determinations of the equation and reading off the corresponding amount of Ea from the total nitrogen curve of the serum (as, for example, Curve IV, Text-fig. 1). The validity of the method is supported by the next to last experiment recorded in Table II, in which it is shown that the presence of super-

<sup>2</sup> Toward the end of this period, when the animal's serum contained  $< 0.2$  mg. of precipitin N per ml., a single injection of 10 mg. of crystalline horse serum albumin was given. Quantitative analyses at short intervals failed to reveal any "anamnestic" rise in anti-egg albumin.

TABLE II

*Addition of Increasing Amounts of Egg Albumin to 1.0 Ml.  
Serum 3.87, 1:1, at 0°*

| Ea N added  | Ea N pptd. | Total N pptd. | Antibody N by difference | Ratio anti-body N:Ea N in ppt. | Anti-body N pptd., calcd. from eq. [3] | Anti-body N pptd., calcd. from eq. [6] | Tests on supernatant             |
|---|------------|---------------|--------------------------|--------------------------------|--|--|----------------------------------|
| mg.   | mg.        | mg.           | mg.                      |                                | mg.                                    | mg.                                    |                                  |
| <b>Course I</b>   |            |               |                          |                                |  |  |                                  |
| Mg. antibody N pptd. = 15.8 Ea N — 83 (Ea N) <sup>2</sup>   |            |               |                          |                                |  |  |                                  |
| Mg. antibody N pptd. = 19.4 Ea N — 36 (Ea N) <sup>3/2</sup> |            |               |                          |                                |  |  |                                  |
| 0.0091  | Total*     | 0.156†        | 0.147                    | 16.2                           | 0.137                                  | 0.146                                  | Excess A                         |
| 0.0155  | "          | 0.236         | 0.220                    | 14.2                           | 0.225                                  | 0.231                                  | " "                              |
| 0.025   | "          | 0.374         | 0.349                    | 14.0                           | 0.343                                  | 0.342                                  | " "                              |
| 0.040   | "          | 0.526†        | 0.486                    | 12.2                           | 0.499                                  | 0.488                                  | " "                              |
| 0.050   | "          | 0.632         | 0.582                    | 11.6                           | 0.582                                  | 0.567                                  | " "                              |
| 0.065   | "          | 0.740         | 0.675                    | 10.4                           | 0.677                                  | 0.664                                  | Excess A, trace Ea               |
| 0.074   | "          | 0.794         | 0.720                    | 9.7                            | 0.714                                  | 0.710                                  | No A or Ea                       |
| 0.082   | "          | 0.830         | 0.748                    | 9.1                            | 0.738                                  | 0.746                                  | No A, < 0.001 Ea N               |
| 0.090   | 0.087      | 0.826         | 0.739                    | 8.5                            | 0.746                                  | 0.763                                  | Excess Ea, analyses in Table III |
| 0.098   | 0.089      | 0.820         | 0.731                    | 8.2                            |  |  | " "                              |
| 0.124   | 0.087      | 0.730         | 0.643                    | 7.4                            |  |  | " "                              |
| 0.135   | (0.072)‡   | 0.610†        | (0.538)                  | (7.5)                          |  |  | " "                              |
| 0.195   | (0.048)    | 0.414         | (0.366)                  | (7.6)                          |  |  | " "                              |
| 0.307   | (0.004)    | 0.106         |                          |                                |  |  | " "                              |
| 0.490   |            | 0.042         |                          |                                |  |  |                                  |

Maximum Ea N, A N in ppt. according to equation [3], 0.095, 0.752; according to equation [6], 0.129, 0.836

**Course II**

Mg antibody N pptd. = 20.4 Ea N — 96 (Ea N)<sup>2</sup>

Mg. antibody N pptd. = 26.1 Ea N — 48.1 (Ea N)<sup>3/2</sup>

|        |          |       |         |       |       |       |                                  |
|--------|----------|-------|---------|-------|-------|-------|----------------------------------|
| 0.0155 | Total*   | 0.306 | 0.290   | 18.7  | 0.293 | 0.312 | Excess A                         |
| 0.050  | "        | 0.844 | 0.794   | 15.9  | 0.780 | 0.766 | " "                              |
| 0.088  | "        | 1.144 | 1.056   | 12.0  | 1.053 | 1.039 | " "                              |
| 0.098  | "        | 1.180 | 1.082   | 11.0  | 1.077 | 1.081 | No A or Ea                       |
| 0.118  | "        | 1.214 | 1.096   | 9.3   |       | 1.127 | " " " "                          |
| 0.127  | "        | 1.278 | 1.151   | 9.1   |       | 1.137 | < 0.001 Ea N                     |
| 0.135  | "        | 1.280 | 1.145   | 8.5   |       | 1.138 | < 0.001 Ea N                     |
| 0.143  | 0.142    | 1.288 | 1.146   | 8.1   |       | 1.133 | Excess Ea, analyses in Table III |
| 0.195  | 0.123    | 1.024 | 0.901   | 7.3   |       |       | " "                              |
| 0.490  | (0.018)  | 0.154 | (0.136) | (7.5) |       |       | " "                              |
| 0.490  | (0.034)§ | 0.154 | (0.120) | (3.5) |       |       | " "                              |

Maximum Ea N, A N in ppt. according to equation [3], 0.106; 1.053; according to equation [6], 0.131, 1.139

\* Assumed.

‡ 3.87 II, 1:1, used.

† At pH 6.36.

§ 3.87 I, 1:1, used.



## QUANTITATIVE THEORY OF PRECIPITIN REACTION. III

TABLE II (concluded)

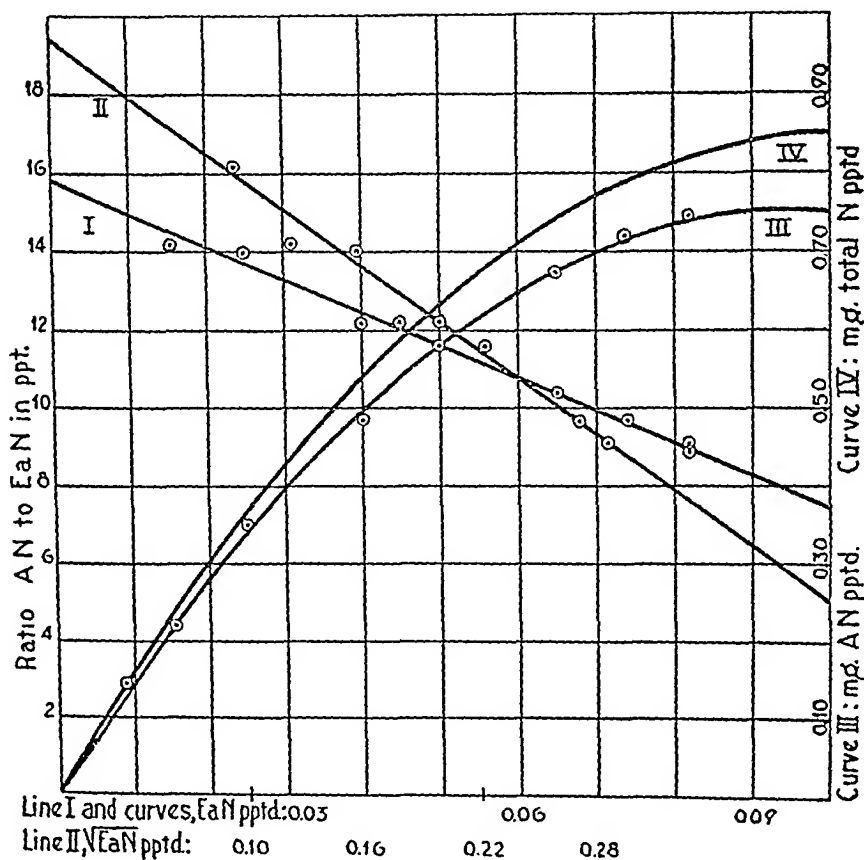
| Ea N added   | Ea N pptd.                      | Total N pptd. | Antibody N by difference | Ratio anti-body N:Ea N in ppt. | Anti-body N pptd., calcd. from eq. [3] | Anti-body N pptd., calcd. from eq. [6] | Tests on supernatant             |
|--|---------------------------------|---------------|--------------------------|--------------------------------|--|--|----------------------------------|
| mg.  | mg.                             | mg.           | mg.                      |                                | mg.                                    | mg.                                    |                                  |
| Course III   |                                 |               |                          |                                |  |  |                                  |
| Mg. antibody N pptd. = $24.8 \text{ Ea N} - 111 (\text{Ea N})^2$   |                                 |               |                          |                                |  |  |                                  |
| Mg. antibody N pptd. = $31.8 \text{ Ea N} - 56.6 (\text{Ea N})^{3/2}$                                      |                                 |               |                          |                                |  |  |                                  |
| 0.0296   | Total*                          | 0.665         | 0.635                    | 21.5                           | 0.622                                  | 0.652                                  | Excess A                         |
| 0.049  | "                               | 1.005         | 0.956                    | 19.5                           | 0.949                                  | 0.944                                  | " "                              |
| 0.079  | "                               | 1.320         | 1.241                    | 15.7                           | 1.267                                  | 1.256                                  | Traces A, Ea (?)                 |
| 0.082  | "                               | 1.370         | 1.288                    | 15.7                           | 1.288                                  | 1.279                                  | Excess A                         |
| 0.088  | "                               | 1.422         | 1.334                    | 15.2                           | 1.324                                  | 1.319                                  | Slight excess A                  |
| 0.098  | "                               | 1.468         | 1.370                    | 14.0                           | 1.364                                  | 1.379                                  | No A or Ea                       |
| 0.122  | "                               | 1.570         | 1.448                    | 11.9                           | 1.373                                  | 1.466                                  | " " " "                          |
| 0.140  | "                               | 1.592         | 1.452                    | 10.4                           |  | 1.487                                  | " " " "                          |
| 0.157  | "                               | 1.606         | 1.449                    | 9.2                            |  | 1.474                                  | " " " "                          |
| 0.195  | 0.194                           | 1.650         | 1.456                    | 7.5                            |  | 1.330                                  | Excess Ea, analyses in Table III |
| 0.234  | 0.202                           | 1.542         | 1.340                    | 6.6                            |  |  | " "                              |
| 0.296  |                                 | 1.025         |                          |                                |  |  |                                  |
| Maximum Ea N, A N in ppt. according to equation [3], 0.112, 1.393; according to equation [6], 0.140, 1.483 |                                 |               |                          |                                |  |  |                                  |
| 0.140  | 0.140 + 3 ml. 0.140 supernatant | 1.542         | 1.402                    |                                |  |  |                                  |
| 0.140 + 3 ml. 0.140 supernatant  |                                 | 1.538         | 1.398                    |                                |  |  |                                  |
| 0.157 + 1.0 ml. supernatant from 3.87 III serial experiment (Table V)                                      | 1.866 Found above               | 1.709         |                          |                                |  |  |                                  |
|  |                                 | 1.449         |                          |                                |  |  |                                  |
|  |                                 | 0.36          |                          |                                |  |  |                                  |

|| 1 determination discarded.

natant from tubes in which maximum precipitation has occurred fails to increase the amount of Ea-A normally precipitated. When it becomes necessary to analyze supernatants containing more Ea, as in the inhibition zone, the presence of dissolved Ea-A introduces complications. These were discussed in Reference 2, and it is accordingly assumed that in the analysis of inhibition zone supernatants for Ea, as well as in the dye-antidye system, all of the dissolved antibody in the

supernatant is precipitated together with that derived from the serum used for the analysis.

The following method of calculation, based on this assumption, gives the same result as the simpler method given at the beginning of the preceding paragraph in the region of antigen excess up to the inhibition zone, and the differences from it are small up to the range in which inhibition is pronounced. In this range the simpler



TEXT-FIG. 1

method soon leads to values of Ea in the supernatant higher than the total added, whereas the method given below gives this result only when inhibition is practically complete, in which case it is necessary to use so small an aliquot that any analytical errors would be greatly multiplied in the final result.

Let A = the maximum antibody nitrogen found in the serum used, Ea = the amount of egg albumin nitrogen added, and N = the amount of nitrogen precipi-

tated at the point considered. Then the amount of specific nitrogen (antigen as well as antibody) in the supernatant is given by  $A + Ea - N$ , and all of this nitrogen would be precipitated in the analysis of the supernatant for Ea with excess antibody according to the quantitative theory elaborated in Reference 1, and as was actually found in the dye-antidye system (2). The additional assumption is made that the entire precipitate obtained in this analysis is of uniform composition; in other words, that the dissolved Ea-A present can combine with A until its composition is the same as that of the Ea-A formed by the free Ea present in its reaction with excess antibody.<sup>3</sup>

If  $N'$  = the nitrogen precipitated in the analysis of the supernatant and  $F$  = the fraction of the supernatant used in the analysis,  $N' - F(A + Ea - N)$  = antibody nitrogen precipitated from the serum used in the analysis. If the curve of antibody N precipitated by Ea from this serum be constructed (as, for example, Curve III, Text-fig. 1), the amount of Ea corresponding to this quantity of antibody N may be read off. The percentage of Ea in this portion of the precipitate may then be calculated according to

$$\frac{Ea\ N\ found \times 100}{Ea\ N\ found + antibody\ N\ found} = \text{per cent Ea N.}$$

Since it was assumed that the entire precipitate contains this proportion of Ea,  $N' \times \text{per cent Ea N}$  thus found  $\div F$  = Ea N in total supernatant, and Ea N originally added *minus* this value = Ea N in the original precipitate.

For example (Tables II and III), 0.124 mg. Ea N precipitated 0.637 mg. N from 1.0 ml. diluted serum 3.87 I,  $A = 0.750$  mg. Then  $0.750 + 0.124 - 0.637 = 0.237$  mg. specific N in supernatant. When 0.75 of the supernatant (actually 1.5 ml.) was set up with 1.0 ml. of the same diluted serum 0.412 mg. N ( $N'$ ) was precipitated. Then  $0.412 - [0.75 \times 0.237] = 0.234$  mg. antibody N pptd. from the serum used for the analysis. From Curve III in Text-fig. 1 it is seen that this corresponds to 0.017 mg. Ea N, and  $\frac{0.017 \times 100}{0.017 + 0.234} = 6.8$  per cent of  $N'$ , or 0.412, = 0.028, and this divided by 0.75 = 0.037 mg. Ea N in the entire supernatant.  $0.124 - 0.037 = 0.087$  mg. Ea N in the original precipitate.

The calculations made as above in the region of excess antigen are given in Table III, in which the data in the first three columns are taken from Table II. The amounts of Ea N precipitated, given in the last column of Table III, and the resulting ratios in the original precipitates are included in Columns 2 and 5 of Table II. Values in parentheses are considered uncertain for reasons given in the discussion.

Table IV is compiled from data given by Culbertson (5) in his Table 3, and from one each of the constant antigen and constant antibody experiments given by Taylor, Adair, and Adair (6), and a comparison is given of the experimental values and those calculated according to [3] and [6] below.

<sup>3</sup> Experiments have shown that the Ea-A precipitate at the antigen-excess end of the equivalence zone can combine with antibody added after precipitation is complete.

Table V shows the result of serial additions of small amounts of egg albumin to several anti-egg albumin sera (*cf.* 1). The data on 10.0 ml. of undiluted serum 3.87 II show a pronounced, but relatively small Danysz effect, while those on the diluted serum, 2.24A, do not show this effect, possibly on account of the small amount of antibody present. A serial experiment on 5.0 ml. of undiluted serum 3.87 III showed that 0.460 mg. Ea N, added in four portions, pre-

TABLE III

*Calculation of Ea N in Precipitate in Region of Antigen Excess*

| Ea N added   | Total N pptd. | Specific N in supernatant (A + Ea - N) | Fraction analyzed | Total N pptd. in analysis of fraction | Less specific N in fraction analyzed | Corresponding Ea N | Per cent Ea N in 2nd ppt.* | Ea N in fraction analyzed | Ea N in entire supernatant | Ea N in ppt. |
|--|---------------|--|-------------------|---------------------------------------|--------------------------------------|--------------------|----------------------------|---------------------------|----------------------------|--------------|
| mg.  | mg.           | mg.                                    |                   | mg.                                   | mg.                                  | mg.                |                            | mg.                       | mg.                        | mg.          |
| Serum 3.87 I, 1:1, maximum antibody N pptd., 0.750 mg.   |               |  |                   |                                       |                                      |                    |                            |                           |                            |              |
| 0.090  | 0.826         | 0.014                                  | 1.75†             | 0.078                                 | 0.053                                | 0.0035             | 6.2                        | 0.0048                    | 0.003                      | 0.087        |
| 0.098  | 0.820         | 0.028                                  | 0.75              | 0.106                                 | 0.085                                | 0.0057             | 6.3                        | 0.0067                    | 0.009                      | 0.089        |
| 0.124  | 0.730         | 0.144                                  | 0.75              | 0.412                                 | 0.304                                | 0.0219             | 6.7                        | 0.0276                    | 0.037                      | 0.087        |
| 0.135  | 0.610         | 0.275                                  | 0.50              | 0.596‡                                | 0.458                                | 0.0257             | 5.3                        | 0.0316                    | 0.063                      | 0.072        |
| 0.195  | 0.414         | 0.531                                  | 0.34              | 0.676                                 | 0.495                                | 0.0395             | 7.4                        | 0.0500                    | 0.147                      | 0.048        |
| 0.307  | 0.106         | 0.951                                  | 0.167             | 0.674                                 | 0.515                                | 0.0418             | 7.51                       | 0.0506                    | 0.303                      | 0.004        |
| Serum 3.87 II, 1:1, maximum antibody N pptd., 1.151 mg.  |               |  |                   |                                       |                                      |                    |                            |                           |                            |              |
| 0.143  | 1.288         | 0.006                                  | 1.75†             | 0.046                                 | 0.035                                | 0.0017             | 4.6                        | 0.0021                    | 0.001                      | 0.142        |
| 0.195  | 1.024         | 0.322                                  | 0.50              | 0.664                                 | 0.503                                | 0.0285             | 5.4                        | 0.0359                    | 0.072                      | 0.123        |
| 0.490  | 0.154         | 1.487                                  | 0.167             | 1.180                                 | 0.932                                | 0.0667             | 6.68                       | 0.0788                    | 0.472                      | 0.018        |
| 0.490  | 0.154         | 1.487                                  | 0.125             | 0.738§                                | 0.552                                | 0.0462             | 7.72                       | 0.057                     | 0.456                      | 0.034        |
| Serum 3.87 III, 1:1, maximum antibody N pptd., 1.456 mg. |               |  |                   |                                       |                                      |                    |                            |                           |                            |              |
| 0.195  | 1.650         | 0.005                                  | 1.50†             | 0.026                                 | 0.018                                | 0.001              |                            | 0.001                     | 0.001                      | 0.194        |
| 0.234  | 1.542         | 0.148                                  | 0.75              | 0.566                                 | 0.455                                | 0.0205             | 4.3                        | 0.0243                    | 0.032                      | 0.202        |

\* 100 times value in Column 7 divided by sum of values in Columns 6 and 7.

† Not run in duplicate.

‡ 3.87 II, 1:1, used.

§ 3.87 I, 1:1, used.

cipitated 9.4 mg. A N, calculated to 5.0 cc. From the equation for 3.87 III, the same relative amount of Ea N, 0.046 mg., added at once to 1.0 ml. of 1:1 serum, is found to precipitate 0.91 mg. A N, or 9.1 mg. from 5.0 ml. undiluted serum—again a definite but small Danysz effect. When the serial experiment on this serum was carried to completion it was found that only 11.34 mg. A N could be precipitated, as against 14.55 mg. which should have been found if all the Ea had been added at once. Thus 22 per cent of the

TABLE IV

Experiments of Other Authors Calculated According to Equations [3] and [6]

| Amt. Ea N<br>added (pptd.)   | Total N pptd.                  | Antibody N<br>pptd.   | Ratio A N: Ea N<br>in ppt. | Antibody N<br>pptd., calcd.<br>from equation [3] | Antibody N<br>pptd., calcd.<br>from equation [6] |
|--|--------------------------------|---|----------------------------|--|--|
| mg.  | mg.                            | mg.   |                            | mg.  | mg.  |
| Culbertson (1935) Table 3.   |                                |   |                            |  |  |
|  |                                | Mg. antibody N pptd. = 17.6 Ea N - 64 (Ea N) <sup>2</sup>     |                            |  |  |
|  |                                | Mg. antibody N pptd. = 22.3 Ea N - 34.8 (Ea N) <sup>3/2</sup> |                            |  |  |
| 0.023  | 0.423                          | 0.400   | 17.4                       | 0.371  | 0.391  |
| 0.045  | 0.678                          | 0.633   | 14.1                       | 0.662  | 0.670  |
| 0.068  | 0.968                          | 0.900   | 13.2                       | 0.901  | 0.899  |
| 0.080  | 1.079                          | 0.999   | 12.5                       | 0.998  | 0.996  |
| 0.097  | 1.207                          | 1.110   | 11.4                       | 1.105  | 1.110  |
| 0.114  | 1.249 (Antigen excess)         |   |                            |  |  |
| 0.143  | 1.320 ( " " , maximum total N) |   |                            |  |  |
| Maximum Ea N, A N in ppt. according to equation [3], 0.138, 1.210; according to equation [6], 0.182, 1.355 |                                |   |                            |  |  |

|  |        |   |      |       |       |
|--|--------|---|------|-------|-------|
| Taylor, Adair, and Adair (1934) Table II, Serum 1754C  |        |   |      |       |       |
|  |        | Mg. antibody N pptd. = 18.2 Ea N - 94 (Ea N) <sup>2</sup>     |      |       |       |
|  |        | Mg. antibody N pptd. = 22.3 Ea N - 40.5 (Ea N) <sup>3/2</sup> |      |       |       |
| 0.0149   | 0.250* | 0.235   | 15.8 | 0.250 | 0.258 |
| 0.0297   | 0.506* | 0.476   | 16.0 | 0.458 | 0.454 |
| 0.045  | 0.677  | 0.632   | 14.0 | 0.629 | 0.616 |
| 0.059  | 0.813  | 0.754   | 12.8 | 0.747 | 0.736 |
| 0.074  | 0.914  | 0.840   | 11.4 | 0.832 | 0.835 |
| 0.089†   | 0.974  | 0.885   | 9.9  | 0.875 | 0.907 |
| Maximum Ea N, A N in ppt. according to equation [3], 0.097, 0.881; according to equation [6], 0.135, 1.003 |        |   |      |       |       |

|   |               |   |      |               |               |
|---|---------------|---|------|---------------|---------------|
| Taylor, Adair, and Adair (1934) Table III, Serum 1754D                                    |               |   |      |               |               |
|   |               | Mg. antibody N pptd. = 16.4 Ea N - 54 (Ea N) <sup>2</sup>     |      |               |               |
|   |               | Mg. antibody N pptd. = 20.6 Ea N - 30.6 (Ea N) <sup>3/2</sup> |      |               |               |
| per ml. serum   | per ml. serum | per ml. serum   |      | per ml. serum | per ml. serum |
| 0.046   | 0.684         | 0.638   | 13.9 | 0.640         | 0.646         |
| 0.051   | 0.743         | 0.692   | 13.6 | 0.697         | 0.698         |
| 0.056   | 0.799         | 0.743   | 13.3 | 0.748         | 0.747         |
| 0.062   | 0.883         | 0.821   | 13.2 | 0.810         | 0.804         |
| 0.070   | 0.949         | 0.879   | 12.6 | 0.883         | 0.874         |
| 0.080   | 1.028         | 0.948   | 11.9 | 0.966         | 0.956         |
| 0.093   | 1.096         | 1.003‡  | 10.8 | 1.058         | 1.153         |
| 0.111   | 1.258         | 1.147   | 10.3 | 1.156         | 1.276         |
| 0.139†  | 1.451         | 1.312   | 9.4  | 1.237         |               |
| Maximum Ea N, A N calcd. from equation [3], 0.152, 1.246; from equation [6], 0.202, 1.384 |               |   |      |               |               |

\* Mean of two determinations.

† Trace of antigen in excess.

‡ From weight of precipitate; omitted in calculation of line according to equation [6].

antibody is not precipitable unless other antibody is present, recalling the behavior of certain antidye sera (2). The concluding data of Table II show, however, that the entire amount of this antibody is carried down if added to a fresh portion of serum and egg albumin. These findings are again referred to in the discussion.

TABLE V

*Serial Addition of Egg Albumin to Various Sera, Calculated to Original Volume*

| Total of successive<br>Ea N additions                                   | Total N pptd.  | Total antibody<br>N pptd. | Ratio A N : Ea N<br>in ppt. | Total antibody N<br>pptd. if added in<br>1 portion (Equation<br>[3], Table II) |
|---|----------------|---------------------------|-----------------------------|--|
| mg.   | mg.            | mg.                       |                             | mg.  |
| 3.87 II, undiluted, 10.0 ml.  |                |                           |                             |  |
| 0.0155  | 0.388 (0.438*) | 0.372 (0.422*)            | 24 (27*)                    |  |
| 0.032   |                | 0.775                     | 24.2                        |  |
| 0.049   |                | 1.130                     | 23.1                        |  |
| 0.067   |                | 1.528                     | 22.8                        |  |
| 0.128   |                | 2.81                      | 22.0                        | 2.53   |
| 0.441   |                | 9.03                      | 20.5                        | 8.06   |
| 0.770   |                | 13.90                     | 18.1                        | 12.86  |
| Mg. antibody N pptd. = 23.4 Ea N - 6.9 (Ea N) <sup>2</sup>              |                |                           |                             |  |
| Mg. antibody N pptd. = 24.9 Ea N - 7.9 (Ea N) <sup>3/2</sup>            |                |                           |                             |  |
| Serum 2.24 A, diluted to approximately same A content as 2.24B, 4.0 ml. |                |                           |                             |  |
| 0.0155  | 0.324          | 0.308                     | 19.9                        |  |
| 0.033   |                | 0.576                     | 17.5                        |  |
| 0.0525  |                | 0.789                     | 15.0                        |  |
| Mg. antibody N pptd. = 22 Ea N - 133 (Ea N) <sup>2</sup>                |                |                           |                             |  |
| Serum 2.24B, partially exhausted, 4.0 ml.                               |                |                           |                             |  |
| 0.0155  | 0.248          | 0.232                     | 15.0                        |  |
| 0.033   |                | 0.464                     | 14.1                        |  |
| 0.0525  |                | 0.687                     | 12.1                        |  |
| Mg. antibody N pptd. = 16.8 Ea N - 90 (Ea N) <sup>2</sup>               |                |                           |                             |  |

\* Assuming solubility of precipitate in serum to be as in saline, approximately 0.005 mg. N per ml. This correction would need to be made only on the first precipitation, since the serum would then be saturated with Ea-A compound.

Text-fig. 2 is derived by putting  $A = 1.00$  in the equations according to [4] in Table II and the portion of Table IV dealing with Taylor, Adair, and Adair's figures. Thus the straight lines in the figure represent a comparison of the sera at a common concentration of 1.00 mg. of antibody N per ml.

The pH of serum 3.87 I was 7.8. 10.0 ml. were diluted to 20.0 ml. with saline and a drop of glacial acetic acid. The pH of the resulting dilution was 6.36.<sup>4</sup> Set

<sup>4</sup> Determined electrometrically by Mr. F. Rosebury, of the Department of Biological Chemistry.

up as in Table II with a number of the same dilutions of Ea, the more acid serum gave identical amounts of N, within the limits of error of the method, as at pH 7.8, even in the inhibition zone (*cf.* 2). Since Marrack and Smith (12) have also failed to observe differences due to pH changes within almost the same limits, the details of the experiment are not given.

In Table VI is contained a compilation of the writers' data relative to the equivalence zone and the equivalence point in the Ea-A system, as well as the found and calculated ratios at the point of maximum antibody precipitation.

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*Ratios of Antibody N: Egg Albumin N in Equivalence Zone and at Maximum Antibody Precipitation*

| Serum    | Ratio at antibody excess end of zone | Calculated equivalence point ratio | Ratio at antigen excess end of zone | Ratio at first maximum pptn. point | Calcd. ratio at maximum, equation [6] |
|----------|--------------------------------------|------------------------------------|-------------------------------------|------------------------------------|---------------------------------------|
| 1.31     |                                      |                                    | 9.2                                 | 9.2                                | 7.2                                   |
| 1.35     | 9.4                                  | 9.0                                | 8.6                                 |                                    | 6.2                                   |
| 1.36     | 9.3                                  |                                    | >7.6                                |                                    | 9.8                                   |
| 1.68     | 11.5                                 | 14.0                               | 11.8                                | 10.5                               |                                       |
| 2.24     | 16.1                                 |                                    |                                     |                                    | 6.6                                   |
| 2.32     | 9.1                                  |                                    | 9.1                                 | 9.1                                | 8.7                                   |
| 3.87 I   | 10.4                                 | 9.8                                | 8.1                                 | 9.1                                | 10.2                                  |
| 3.87 II  | (11.5)                               | 9.8                                | 7.5                                 | 11.9                               |                                       |
| 3.87 III | (15.0)                               | 11.3                               |                                     |                                    |                                       |

Values in parentheses are estimated from nearest actual determination.

#### DISCUSSION

That crystalline egg albumin is a single definite chemical individual appears to be established with as great certainty as possible in case of a protein. When injected into rabbits it nevertheless rise to antibodies of differing reactivity, as has already been noted by Hooker and Boyd (13), and as is shown in the present communication. In spite of this it is assumed in the following discussion, in References 1 and 2, that the average behavior of the antibodies is that of a single substance, and that it may accordingly be treated mathematically as such.

Another assumption, which was made in Reference 1 but which was not applicable in Reference 2, was that in the region of excess antigen all of the hapten (antigen) added was precipitated since non-

detected in the supernatant by the exceedingly delicate serological test. The same assumption, which is not considered valid by Taylor, Adair, and Adair (6), is made in the egg albumin (Ea)-antibody (A) system, for the following reasons.

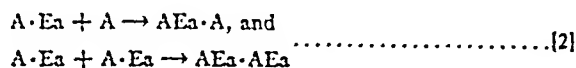
(a) The antigen is a single definite chemical individual. (b) The test for Ea with homologous antibody is sensitive to an antigen dilution of at least 1:200,000, and the dissociation constant of a soluble Ea-A complex would have to be exceedingly small if the Ea in it were to escape detection. (c) When soluble Ea-A compounds are known to be present, as in the inhibition zone, these are precipitated when fresh antibody is added. (d) In a serial experiment, such as the first in Table V, antigen appears in excess as soon as all but the last traces of antibody precipitable in this way have been thrown down. The serially non-precipitable antibody can thus scarcely be concerned in the disappearance of Ea into an undissociated, soluble Ea-A complex, the more so as it is quantitatively precipitated when added to fresh antibody and Ea (see last experiment in Table II). A third type of antibody would then have to be postulated. (e) If it is assumed that all of the Ea added in the region of excess antibody is precipitated, the resulting A N:Ea N ratios in the precipitate and the range of these ratios with varying Ea-A proportions are found to be of the same magnitude as was observed in the dye-antidye system (2) in which antigen and antibody in the precipitate were each directly determined.

As in the instances studied in the first two papers of this series (1, 2) it is considered that the precipitin reaction between crystalline egg albumin and antibody occurs in steps in a series of bimolecular reactions which take place before precipitation begins.

The first step in the reaction would be



This would represent the equivalence point compound in its simplest form, as composed of 1 unit of A and 1 unit of Ea, regardless of their actual molecular proportions. A molecular formula would be  $A_m Ea_n$ , and this compound would be arrived at through a series of bimolecular reactions. Since both A and Ea are proteins, and the opportunity is given for the immunologically reactive groupings to recur a number of times, the reactants may be considered multivalent with respect to each other. Thus the  $A \cdot Ea$  compound initially formed could react with other molecules of the same compound, or with A or Ea, if either is present in excess. In the region of excess antibody the second step of the reaction would then consist of the two competing bimolecular reactions, in which dissociation is assumed to be negligible:





up as in Table II with a number of the same dilutions of Ea, the more acid serum gave identical amounts of N, within the limits of error of the method, as at pH 7.8, even in the inhibition zone (*cf.* 2). Since Marrack and Smith (12) have also failed to observe differences due to pH changes within almost the same limits, the details of the experiment are not given.

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| 1.35     | 9.3                                  |                                    | 8.6                                 |                                    | 6.2                                   |
| 1.36     | 11.5                                 |                                    | >7.6                                |                                    | 9.8                                   |
| 1.68     | 16.1                                 | 14.0                               | 11.8                                | 10.5                               |                                       |
| 2.24     | 9.1                                  |                                    |                                     |                                    | 6.6                                   |
| 2.32     | 10.4                                 | 9.8                                | 9.1                                 | 9.1                                | 8.7                                   |
| 3.87 I   | (11.5)                               | 9.8                                | 8.1                                 | 11.9                               | 10.2                                  |
| 3.87 II  | (15.0)                               | 11.3                               | 7.5                                 |                                    |                                       |
| 3.87 III |                                      |                                    |                                     |                                    |                                       |

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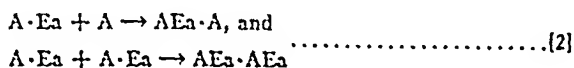
(a) The antigen is a single definite chemical individual. (b) The test for Ea with homologous antibody is sensitive to an antigen dilution of at least 1:200,000, and the dissociation constant of a soluble Ea-A complex would have to be exceedingly small if the Ea in it were to escape detection. (c) When soluble Ea-A compounds are known to be present, as in the inhibition zone, these are precipitated when fresh antibody is added. (d) In a serial experiment, such as the first in Table V, antigen appears in excess as soon as all but the last traces of antibody precipitable in this way have been thrown down. The serially non-precipitable antibody can thus scarcely be concerned in the disappearance of Ea into an undissociated, soluble Ea-A complex, the more so as it is quantitatively precipitated when added to fresh antibody and Ea (see last experiment in Table II). A third type of antibody would then have to be postulated. (e) If it is assumed that all of the Ea added in the region of excess antibody is precipitated, the resulting A N:Ea N ratios in the precipitate and the range of these ratios with varying Ea-A proportions are found to be of the same magnitude as was observed in the dye-antidye system (2) in which antigen and antibody in the precipitate were each directly determined.

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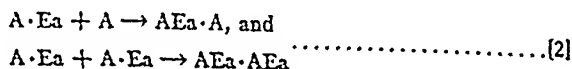
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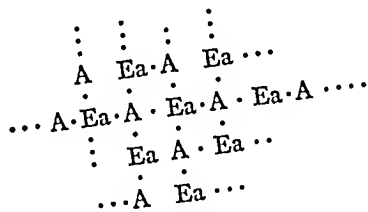
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Since both A and Ea may be considered multivalent with respect to each other, the products of reaction [2] would combine chemically until aggregates large enough to separate from solution are formed. This might be represented two-dimensionally as follows:



in which the end-result would be much the same as that pictured by Marrack (14).

In the special case in which A and Ea are mixed in equivalent proportions the A·Ea (or  $A_m Ea_n$ ) produced according to [1] would merely polymerize, and the equivalence point precipitate would be  $(A \cdot Ea)_x$ , or  $(A_m Ea_n)_x$ .

As in the reactions studied in References 1 and 2 the composition of the precipitate would thus depend on the relative proportions in which the reactants are mixed. As in Reference 2, only a small proportion of the antibody was found to react to form compounds containing more A than twice the equivalence point ratio, so that the additional reactions which it was necessary to consider in Reference 1 may be neglected. Based on the above concept of the reaction the following expression for the region of excess antibody may be derived with the aid of the mass law as in Reference 1:

$$\text{mg. antibody N precipitated} = 2R(Ea \text{ N}) - \frac{R^2(Ea \text{ N})^2}{A} \dots\dots\dots [3]$$

in which  $R$  = the ratio of antibody nitrogen to egg albumin nitrogen in the precipitate at the point at which antigen first appears in excess;  $Ea \text{ N}$  = egg albumin nitrogen precipitated, and  $A$  = the amount of antibody N precipitated at the point at which antigen first appears in excess. Only in serum 3.87 III was  $R$  in better agreement with the equivalence point than the point chosen (cf. also Table VI).

The Ea-A system differs from the dye-antidye and S III-antibody systems already studied in that maximum precipitation of antibody is reached at a point very close to that at which antigen first appears in excess, after which inhibition of specific precipitation begins very rapidly. The range of maximum precipitation in the region of antigen excess is thus so small that the use of an expression such as [4]

in Reference 2 was found unnecessary in the sera studied. Since anti-Ea sera are thus extremely sensitive to an excess of antigen it is necessary to use great care that only a very slight excess of Ea be present in analyzing for maximum antibody precipitation. The amount of Ea to be used may be determined by a rapid preliminary experiment on 0.5 ml. of serum at 37°, in which 0.1 mg. portions of Ea (or less in the case of weak antisera) are added until no further precipitation occurs.

No attempt is made in the present communication to formulate the reaction in the inhibition zone on account of the uncertainties of analysis discussed below. As in the dye-antidye system the relative proportions of the components appear to be the determining factors since precipitation does not occur in the supernatant when the concentration of Ea is reduced by dilution with saline.

From Table I it is seen that precipitation in the Ea-A system is complete in 1 hour at 37°C., and that the amount of nitrogen precipitated is only slightly, if at all less, than at 0° for 48 hours. In one experiment in the inhibition zone, however, with 0.305 mg. Ea N, 0.19 mg. N was precipitated at 37°, 0.12 mg. at 20° for 2 hours and overnight in the cold, and only 0.11 mg. at 0° for 4 days, a result consistent with increased dissociation of the soluble Ea-A compound at the higher temperatures. Except in the inhibition zone, then, there are no marked differences except in rate when the reaction is carried out at 37°, at 0°, or, as in ordinary immunological practice, at 37° for 2 hours and overnight in the cold. Numerous data obtained at 37° are accordingly omitted. Since certain sera show slightly more precipitable nitrogen at 0° for 48 hours than under other conditions this procedure was adopted in the present work, except that experiments in the inhibition zone were allowed to stand for 4 days, with thorough mixing each day. It will also be noted from Table I that the solubility of the specific precipitate up to the region of slight antigen excess is about 0.005 mg. per ml. at 0°, a value somewhat lower than that given by Marrack and Smith (12) for the pseudoglobulin-antibody precipitate. If this value be accepted it would be necessary to correct all values for antibody nitrogen given in this paper by addition of 0.02 or 0.03 mg. of N. Since, however, actual experimental data are presented the correction is indicated only in the case of a serial experiment with 10.0 ml. of serum (Table V), in which enough Ea-A compound is dissolved in the first supernatant (0.05 mg. N, calculated) to make the initial precipitate smaller in amount than the second.

The linear relation

$$\frac{\text{Antibody N}}{\text{Ea N}} \text{ in the precipitate} = 2R - \frac{R^2}{A} (\text{EaN}) \dots\dots\dots [4]$$

follows from equation [3]. By plotting the values of the ratio  $\frac{\text{Antibody N}}{\text{Ea N}}$  against Ea N precipitated a straight line results from which the values of the constants in equations [4] and [3] may be obtained. Thus, in Text-fig. 1, Line I is plotted in this way from data in Table II up to the region of excess antigen. For serum 3.87 I, 1:1, the intercept on the y-axis,  $2R = 15.8$ , and the slope of the line,  $-\frac{R^2}{A} = -83$ , whence  $R = 7.9$ ,  $A = 0.752$ . The experimentally found

values are  $R = 9.1$  and  $A = 0.748$  at the antigen excess end of the equivalence zone. Equation [3] for serum 3.87 I, 1:1, is thus  $N = 15.8 \text{ Ea N} - 83 (\text{Ea N})^2$ , and is represented by Curve III in Text-fig. 1, on which the circles show the experimentally determined points. A comparison of the fourth and sixth columns of Table II shows, in general, close agreement between the experimentally determined values of antibody N precipitated and those calculated according to [3] for the three sera given in the table. Curve IV represents the corresponding total nitrogen curve,  $N = 16.8 \text{ Ea N} - 83 (\text{Ea N})^2$  for serum 3.87 I, 1:1.

Since [4] is a linear expression, the equation for the line may be approximately fixed for any serum by determination (in duplicate) of the amounts of antibody nitrogen precipitated at two points in the region of excess antibody. The greater the number of points determined the greater would be the accuracy attained. As recommended in References 1 and 2, more than one-third of the antibody should be precipitated in order to minimize the error due to the small portion of antibody yielding compounds of ratio  $> 2R$ .

In Reference 2 it was shown that if  $\frac{\text{Antibody N}}{\text{Dye N}}$  in the precipitate were plotted against the square root of dye N precipitated an even closer approximation to a straight line was obtained than by means of an equation of the type of [4].

Reduced to the same form as [7] in Reference 2, this gives

$$\frac{\text{Antibody N}}{\text{Ea N}} \text{ in the precipitate} = 3R'' - 2 \sqrt{\frac{(R'')^2 (\text{Ea N})}{A}} \dots\dots\dots [5]$$

in which  $3R''$  is the intercept on the y-axis and  $-2 \sqrt{\frac{(R'')^2 (\text{Ea N})}{A}}$  is the slope of the line,  $A = \text{maximum precipitable antibody N}$ , and  $R'' = \text{Antibody N} : \text{Ea N}$

ratio at the maximum. For serum 3.87 I, 1:1, the best equation for this line, (Line II, Text-fig. 1), arrived at by application of the method of least squares to the experimental data, is  $\frac{\text{Antibody N}}{\text{Ea N}}$  in the precipitate =  $19.4 - 36 \sqrt{\text{Ea N}}$ .

Then

$$\text{mg. antibody N precipitated} = 19.4 \text{ Ea N} - 36(\text{Ea N})^{3/2} \dots \dots \dots [6]$$

and when the first derivative,  $19.4 - 54 (\text{Ea N})^{1/2} = 0$ , antibody N precipitated at the maximum, or A, = 0.836, a value somewhat higher than that actually found, 0.748. R'', also, = 6.5, which is too low. It will be noted, however, from the maxima and from the values calculated according to equation [6] given in Column 7 of Table II, that this relation gives better agreement with the experimental values for serum 3.87 II than does equation [3], while in the case of serum 3.87 III both methods of calculation fit the data well.

In Table IV are given data from papers by Culbertson (5) and Taylor, Adair, and Adair (6) as far as the region of antigen excess, and it is seen that the Ea-A reaction in the sera studied by these workers may also be quantitatively expressed up to this region according to equation [3] which follows from the writers' theory of the reaction, or by the empirical relation [6].

Of the eleven sets of data given by Taylor, Adair, and Adair, only two fail to conform to equation [3] and three to equation [6]. Both of these equations may be used for total N precipitated, in which case the coefficient of the first term is increased by 1, since the total N:Ea N ratio is 1 greater than the A N:Ea N ratio. This was done in calculating Curve IV, Text-fig. 1. In comparing the values calculated according to [3] or [6] with Taylor, Adair, and Adair's data it should also be borne in mind that the first point of maximum antibody precipitation (with increasing amounts of antigen) need not coincide with the point of maximum total nitrogen, since antibody in this range is capable of combining with still more antigen, as shown by the decreasing ratios for A N:Ea N in the precipitate in this range and in the initial portion of the inhibition zone.

It is felt that the inhibition zone data in the Ea-A system offer too many uncertainties to warrant treatment of this portion of the reaction range as in References 1 and 2. The method of calculation of the composition of the precipitate given in the Experimental part (pages 715 and 716) appears to be the most reasonable, but it is not considered entirely trustworthy since it tends to show somewhat



higher amounts of Ea in the supernatants near the region of total inhibition than were actually added. There is an uncertainty as to what correction to apply in order to avoid this.

If, for example, one deducts from the nitrogen precipitated in the analysis of the supernatants the average discrepancy noted in Reference 2, 0.03 mg. N for precipitates of 0.300 mg. N and over, one obtains decreasing ratios for A N:Ea N precipitated in the inhibition zone which agree well with comparable, directly determined ratios in the inhibition zone of the dye-antidye system. However, it is not known whether a like discrepancy, to be corrected for, exists in the Ea-A system, and indeed, the next to last experiment in Table II would seem to argue against such a correction. The uncorrected ratios appear to become constant before inhibition is greatly advanced, and while this result is not improbable, the precipitates become more and more gelatinous as the amounts of Ea added are increased and inhibition becomes more complete, so that a change in composition seems indicated if A in this region may be considered as a single substance. It is hoped to eliminate these uncertainties in work now under way.

The serial experiments shown in Table V, as well as others which are not reported, also appear to conform to equations [3] and [6]. In addition they furnish evidence against the homogeneity of anti-egg albumin. Thus serum 2.24 B, exhausted by Ea additions to approximately the same A content as diluted serum 2.24 A, yielded an equation with entirely different constants (Table V).<sup>5</sup> In general, the Danysz effect is much smaller than would have been expected by analogy with References 1 and 2. In spite of this a fraction of the antibody in sera 2.24 and 3.87 III, the only ones studied from this standpoint, was found to be non-precipitable when the Ea was added in serial portions. This residual antibody, amounting to 22 per cent in the case of serum 3.87 III, might be considered to be of lower reactivity owing to the small number, possibly only one, of immunologically reactive groupings in an otherwise unaltered globulin molecule. Antibody of this type could scarcely build up the large A·Ea aggregates postulated by the writers and by Mar-rack as necessary before precipitation occurs. However, when multivalent (or average) antibody is present any A·Ea compound formed

<sup>5</sup> Similarly, the supernatant from the serial experiment on serum 3.87 II, containing approximately the same amount of precipitin N as 3.87 I, gave a linear equation almost coinciding with the line for 3.87 I, Text-fig. 1. It will be noted, however, from Text-fig. 2 that the equations for the whole sera do not coincide at equal A content.

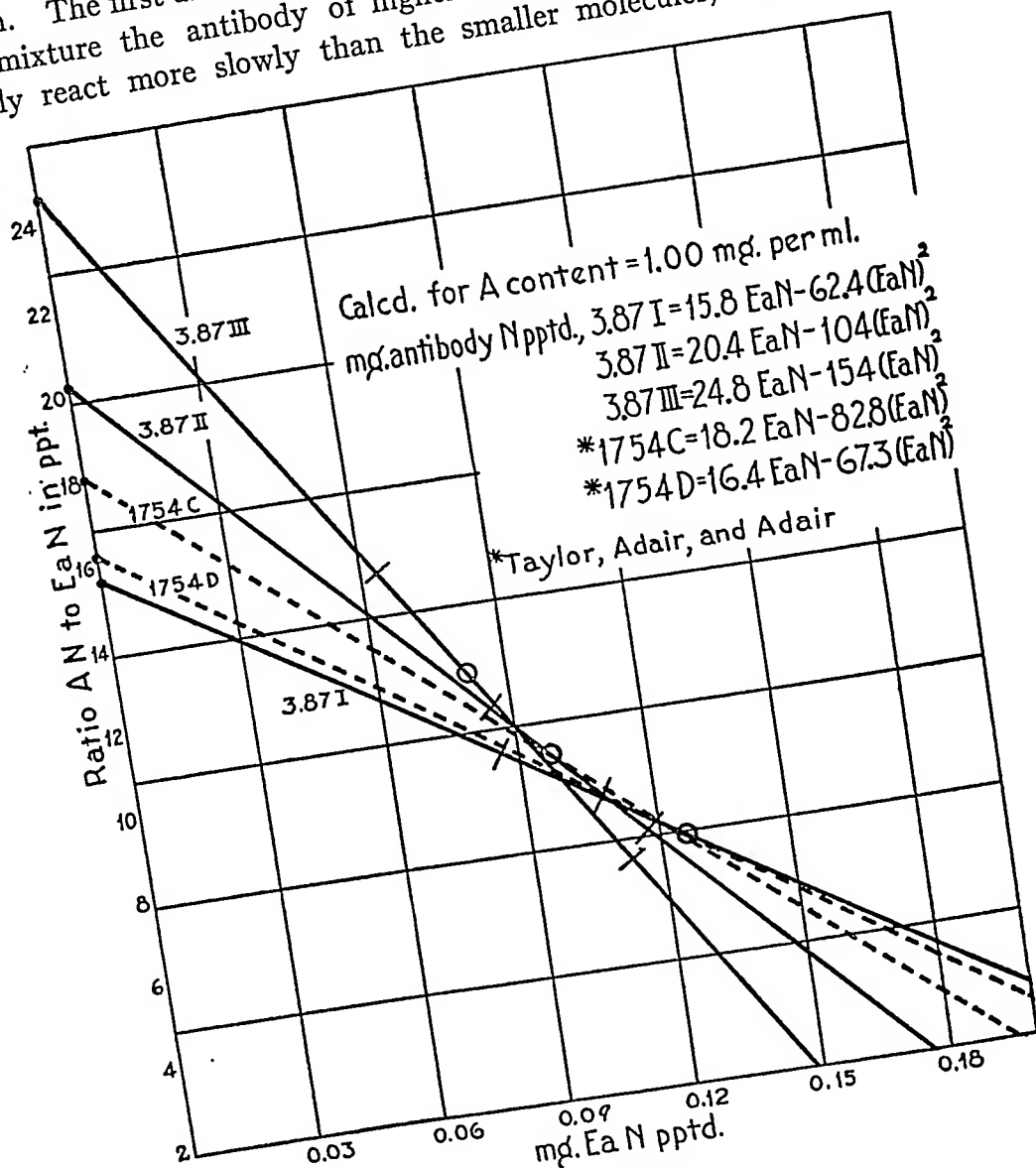
by the residual antibody would participate in the building up of aggregates through the multivalent Ea present and the maximum titer would be attained if sufficient Ea were added, as is actually found. Moreover, if this interpretation be correct, it would be expected that if such low-grade or residual antibody were added in suitable amount to serum before precipitation with a given quantity of Ea, the entire amount of residual antibody would be found added to the precipitable nitrogen obtainable from the serum and Ea alone. That this actually occurs, is seen in the last experiment given in Table II and on pages 717 and 719. The calculated residual antibody N in 1.0 ml. of the serial supernatant, allowing for the successive dilutions necessary, was 0.36 mg., and precisely this amount was found over the normally precipitated N when 1.0 ml. of supernatant was added to serum and Ea.

Thus by means of the quantitative method it has been possible to show, in an antiserum to a single protein antigen, the presence of a low-grade fraction of the total antibody, non-precipitable by the Ea when isolated, but precipitated with the remainder of the antibody under ordinary conditions. A simple explanation of the behavior of this antibody is also offered.

In Text-fig. 2 the lines given by equation [4] for the bleedings from Courses I, II, and III of Rabbit 3.87, (solid lines) and from the third and fourth bleedings of Rabbit 1754 (broken lines, Taylor, Adair, and Adair) are compared at 1.00 mg. of antibody N per ml.

It will be noted that all points up to the calculated maximum (indicated by the circle) on the line for 3.87 III lie above those of 3.87 II, which in turn lie above those of 3.87 I. The same result holds if the found maxima are taken as points of reference. Thus the antibody, considered as a single reactive unit, has become capable of combining with Ea to form compounds of higher A N: Ea N ratio in each successive bleeding. Two readily occurring explanations of this are: that antibody of higher and higher molecular weight, with the same combining capacity, is formed as the immunization proceeds, or that in the later stages antibody is formed which is reactive with a larger number of chemically distinct groupings on the Ea molecule than was the antibody produced in the earlier stages of immuniza-

tion. The first alternative is believed the less probable, for in such a mixture the antibody of higher molecular weight would probably react more slowly than the smaller molecules, and the pre-



TEXT-FIG. 2

precipitates formed with small amounts of Ea would have lower A N: Ea N ratios than those formed with larger amounts of Ea.<sup>6</sup> The <sup>6</sup> This is contrary to the experimental findings, including those summarized in footnote 5.

second explanation is also in accord with the broadening of the scope of cross-reactions so often observed on continued immunization (for references, *cf.* (15)). At any point in the region of antibody excess a given amount of antibody 3.87 I combines with more Ea than does 3.87 II, and this, in turn combines with more than does 3.87 III: another way of expressing the relation noted above. This does not mean, however, that the same relative efficiency obtains at other regions in the reaction range; thus, at the antigen excess end of the equivalence zone, the last point at which all of the Ea added is precipitated, 1.00 mg. of antibody I, II, and III combines with 0.110, 0.123, and 0.133 mg. of Ea N, respectively. It is evident from these considerations and the results of References 1 and 2 that, in general, a given amount of antibody combines with the largest amount of antigen, or shows its greatest efficiency, toward the region of antigen excess. Since excess of antibody is approached in therapy through the region of antigen excess, it is possible that a number of small injections of serum over a short period might have a greater effect than a single large injection.

In the third (C) and fourth (D) bleedings of Rabbit 1754 reported by Taylor, Adair, and Adair and shown as the dotted lines in Text-fig. 2, it would appear that the antibody produced by this animal at the third bleeding was capable of reacting with more groupings on the Ea molecule than was that of the fourth bleeding, although the difference was not large. Attempts will be made to study these relationships in a series of animals from the first appearance of precipitin through a long series of courses.

In Text-fig. 2 the perpendicular lines mark the extent of the equivalence zone in each case, as also shown in Table VI. It will be noted that this zone was most limited in the earliest serum, was somewhat broader in II, although the equivalence point (taken as the mean of the ratios at the ends of the zone) remained the same, and was of such extent in III that the calculated equivalence point, though higher, scarcely has any real meaning. In this serum, tests for A and Ea in the supernatant were negative over a twofold range of concentration of Ea.

The broadening of the equivalence zone on continued immunization is also in accord with the conception that antibody formed in the later stages is reactive with an increased number of chemically distinct groupings on the Ea molecule.

As had been found in References 1 and 2 the data in Table VI indicate that it is a matter of considerable difficulty to fix accurately the limits of the equivalence zone so that the equivalence point may be even approximately calculated. In most of the anti-Ea sera studied by the writers, by Culbertson (4, 5), and by Taylor, Adair, and Adair (6), the equivalence zones extended over a considerable range, and the extremes of the approximate equivalence points differed by as much as 50 per cent of the lower values. Consequently, this point can scarcely be said to be "constant" as considered by the other workers and by Hooker and Boyd (7). While the average equivalence point ratio appears to be fairly characteristic for any antigen-antibody system, as shown by Hooker and Boyd, the variations of the equivalence point ratio in any one system are so great, even in different bleedings from the same animal, as to render it likely that this ratio is governed by other factors as well as the relative molecular weights of the reactants. It would appear to the writers that available evidence favors the view that combining ratios over the entire range of the precipitin reaction depend on the relative numbers of reactive groupings in the antigen and antibody molecules as well as on the molecular weights.

The parallel study of the constant-antigen and constant-antibody series made by Taylor, Adair, and Adair (6) indicates, as these workers and others have pointed out, the probable correspondence of the constant-antibody flocculation optimum with the equivalence point; the chemical significance of the constant-antigen optimum is not clear. Although titrations made in these two ways show optimal ratios with the quotient 1.6, experiments of both types yield similar relations when calculated according to equations [3] or [6], as shown in Table IV.

In conclusion it is again pointed out that evidence is presented in References 1 and 2 and the present paper that antibody is not homogeneous. Much of this evidence, it is believed, could not have been secured by the use of any but an accurate, quantitative method. At any rate it is apparent that a theory based on the statistical behavior of antibody as a single substance can serve merely as a temporary expedient, useful in its application to antisera as they

occur, and until such time as it may be possible to isolate antibody possessed of a single reactivity.

#### SUMMARY

1. A quantitative theory of the precipitin reaction based on the laws of classical chemistry has now been found applicable to the crystalline egg albumin-antibody system. Equations derived from the theory permit the calculation of the behavior of an anti-egg albumin serum over most of the reaction range after a few quantitative analyses have been made for the nitrogen precipitated. Data of other workers also conform to the proposed equations.

2. The empirical relation, shown to have advantages in the dye-antidye system, may also be used for the Ea-A reaction.

3. Serum from the same animal after successive courses exhibits progressive changes which have been described graphically and quantitatively. These changes are believed to consist in the formation of more and more antibody capable of reacting with a larger number of chemically different groupings in the antigen molecule.

4. Evidence is presented that anti-egg albumin is not homogeneous, and that even after prolonged immunization the antiserum contains much low-grade antibody, incapable of forming precipitates unless more reactive precipitin is present.

5. Factors affecting the equivalence point ratio are discussed.

In conclusion the writers wish to express their thanks for assistance given by Dr. Torsten Teorell.

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# FURTHER OBSERVATIONS ON THE BLOOD CHOLESTEROL OF RABBITS IN RELATION TO ATHEROSCLEROSIS\*

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It was reported previously (1) that dried whole thyroid or potassium iodide administered concurrently with cholesterol to rabbits prevented the usual hypercholesterolemia and atherosclerosis. Thyroxin seemed less effective, and the bromide and chloride of potassium were ineffective. The protective action of the iodide was abolished by removal of the thyroids (2). Since these reports, further observations have been made on cholesterol-fed rabbits, the results of which are given herewith.

Rosenthal (3) found that rabbits fed cholesterol and small amounts of iodide actually developed a higher blood cholesterol and more marked atherosclerosis than those animals fed cholesterol alone. The amount of iodide given was calculated from a human therapeutic dose on the basis of comparative weights. It was pointed out quite rightly that, were the amount of iodide necessary to prevent hypercholesterolemia in rabbits translated proportionally into terms of human therapy, severe iodism would undoubtedly result. Rabbits, however, are notably tolerant to iodine, remaining in good condition even on huge doses. Nor does it seem with our present knowledge that results obtained in herbivores can be applied through inference to human beings or *vice versa*.

Page and Bernhard (4) also found that rabbits fed cholesterol and an organic iodide developed an average plasma cholesterol higher than those fed cholesterol alone. The animals given iodide, however, were largely protected from atherosclerosis.

The apparent discrepancy in the action of iodine upon the level of cholesterol in the blood has been explained to a large degree in an interesting report by Breusch and Thiersch (5). These investigators found that the effect of iodine depended upon the dose. Large doses of iodide entirely prevented a rise in the blood cholesterol while small amounts actually augmented this rise in cholesterol-fed rabbits.

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*Methods*

The rabbits used in this work were mostly of the Dutch belted variety. They were 4 to 6 months old when received unless otherwise noted. The animals were kept indoors in individual cages. The diet consisted of oats and fresh vegetables. Each rabbit was given 1 gm. of crystalline cholesterol mixed with the grain three times a week.

Blood was obtained from the ear vein at 7 or 10 day intervals. Cholesterol determinations were made on the whole blood by the method of Bloor, Pelkan and Allen (6).

*Duration of Protective Action of Potassium Iodide*

Although potassium iodide was effective in preventing a rise in the blood cholesterol when fed in large amounts with cholesterol to rabbits

TABLE I  
*Duration of Protective Action of Potassium Iodide*

| TABLE I   |        |     |     |     |     |     |     |     |     |     |     |
|---|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Duration of Protective Action of Potassium Iodide |        |     |     |     |     |     |     |     |     |     |     |
| Blood cholesterol, mg. per 100 cc.                |        |     |     |     |     |     |     |     |     |     |     |
| Rabbit No.  | Months |     |     |     |     |     |     |     |     |     |     |
|   | 0      | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
| • 2-35  | 123    | 189 | 167 | 222 | 166 | 219 | 384 | 521 | 497 | 548 | 503 |
| 2-36  | 113    | 151 | 133 | 161 | 169 | 158 | 182 | 282 | 444 | 495 | 475 |
| 2-37  | 123    | 149 | 143 | 138 | 184 | 192 | 237 | 239 | 321 | —   | —   |
| 2-38  | 123    | 146 | 120 | 137 | 170 | 216 | 226 | 303 | 184 | 141 | 200 |
| 2-39  | 108    | 125 | 123 | 143 | 162 | 247 | 251 | 238 | 382 | 393 | 296 |
| Average.....                                      | 118    | 152 | 137 | 160 | 170 | 206 | 256 | 317 | 366 | 394 | 369 |

(2) it was noted that, toward the end of the feeding period, which was arbitrarily set at 110 days, there was a tendency for the level of cholesterol in the blood to rise. This led to the conjecture that, possibly, the protective action of the iodide was impermanent and that, after a time, the animal might "escape" from the iodide effect resulting in the development of a hypercholesterolemia.

To test this possibility rabbits were fed cholesterol and potassium iodide over a period of 10 months.

Five rabbits were used. There were 3 males and 2 females. Cholesterol was given as described above. Each animal also received 1 gm. of potassium iodide

in aqueous solution three times a week mixed with the grain. Blood cholesterol determinations were made every 10 days.

Rabbit 2-37 died toward the end of the 8th month and was not autopsied. The other 4 animals remained in good health and were killed after 10 months. At autopsy all showed atheromatous changes in the aorta ranging from slight to marked in degree. In the gross there was an increase in liver fat in 3.

The blood cholesterol values are shown in Table I. For the sake of conciseness the average of the three monthly readings is given in each instance.

The iodide exerted a protective action and prevented a significant rise in the blood cholesterol for the first 4 months. Thereafter it seemed to lose its effectiveness, and the blood cholesterol mounted steadily throughout the remaining 6 months, much as it does in rabbits fed cholesterol alone.

This loss of effectiveness is curious and unexplained.

#### *Effect of Potassium Iodide after Prolonged Cholesterol Feeding*

Up to this point the work had been concerned with the effect of various substances on the blood cholesterol of rabbits when given at the beginning of the cholesterol feeding. It was now of interest to determine the effect of potassium iodide on the hypercholesterolemia of rabbits that had been fed cholesterol for a long time.

Nine rabbits were available that had been given cholesterol continuously for 11 months. Experience has shown that rabbits fed for as prolonged a period as this should be thoroughly "saturated" with cholesterol. If the animals were autopsied, marked atheromatous lesions in the arteries and extensive fatty deposits in the viscera presumably would be found.

The cholesterol feeding was continued. In addition, each animal was given a gram of potassium iodide thrice weekly for a month.

The blood cholesterol values are shown in Table II, and are expressed graphically in Fig. 1. The first five determinations given in the table were made during the 10th and 11th months of cholesterol administration and serve as a base line for comparison with the effect of the iodide. Essentially similar figures were obtained during previous months of feeding but have been omitted as irrelevant.

It is apparent that the administration of the potassium iodide produced a sharp rise in the already high blood cholesterol level. When the iodide was stopped the cholesterol values fell again, less abruptly than they had risen, and were approaching the former base line when

## BLOOD CHOLESTEROL AND ATHEROSCLEROSIS

TABLE II  
Effect of Potassium Iodide after Prolonged Cholesterol Feeding

| Rabbit No.   | Blood cholesterol, mg. per 100 cc. |     |     |     |     |     |     |      |                      |     |     |     |      |     |   |   |
|--------------|------------------------------------|-----|-----|-----|-----|-----|-----|------|----------------------|-----|-----|-----|------|-----|---|---|
|              | 10 day periods                     |     |     |     |     |     |     |      |                      |     |     |     |      |     |   |   |
|              |                                    |     |     |     |     |     |     |      | Potassium iodide fed |     |     |     |      |     |   |   |
| 1-90         | 730                                | 682 | 636 | 766 | 670 | 832 | 750 | 872  | 682                  | 625 | 568 | 647 | 595  | —   | — | — |
| 1-92         | 432                                | 399 | 528 | 486 | 514 | 514 | 686 | 615  | 586                  | 832 | 892 | 854 | 1042 | —   | — | — |
| 1-94         | 647                                | 658 | 682 | 636 | 780 | 736 | 961 | 1013 | 1102                 | 782 | 720 | 781 | 750  | 646 | — | — |
| 1-95         | 421                                | 338 | 389 | 361 | 425 | 507 | 586 | 560  | 698                  | 638 | 487 | 577 | 568  | 500 | — | — |
| 2-01         | 475                                | 568 | 568 | 528 | 529 | 721 | 708 | 1137 | 1102                 | 782 | 735 | 593 | —    | —   | — | — |
| 2-02         | 353                                | 463 | 413 | 379 | 350 | 329 | 568 | 658  | 503                  | 577 | 395 | 452 | 470  | 521 | — | — |
| 2-04         | 239                                | 310 | 298 | 291 | 293 | 310 | 335 | 357  | 338                  | 347 | 417 | 500 | 458  | —   | — | — |
| 2-05         | 446                                | 357 | 264 | 395 | 417 | 441 | 658 | —    | 708                  | 765 | 625 | 514 | 368  | 436 | — | — |
| 2-06         | 399                                | 289 | 318 | 248 | 268 | 290 | 521 | —    | 544                  | 576 | 586 | 354 | 399  | 371 | — | — |
| Average..... | 460                                | 452 | 455 | 454 | 472 | 520 | 641 | 745  | 696                  | 658 | 603 | 586 | 581  | 495 | — | — |

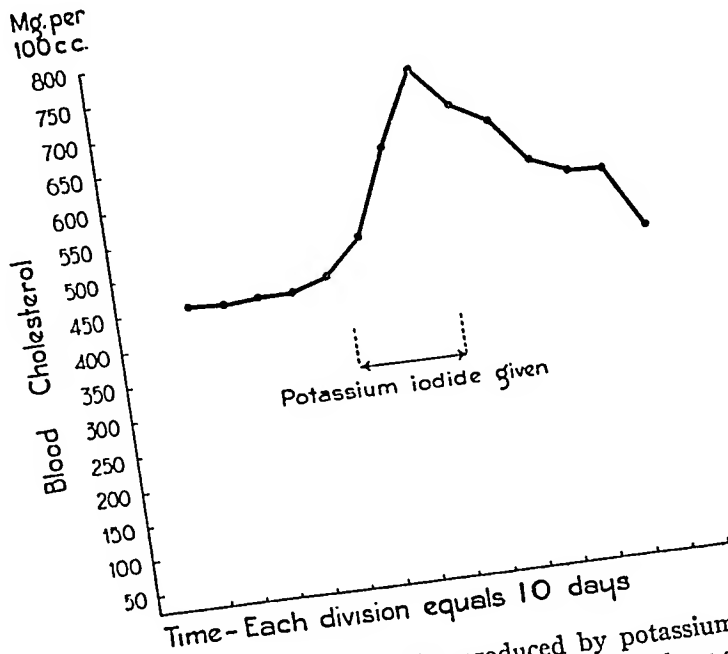


FIG. 1. Curve showing the sharp rise produced by potassium iodide in the average blood cholesterol values of a group of rabbits with long standing hypercholesterolemia.

an epidemic of "snuffles" killed 5 of the rabbits and ended the experiment.<sup>1</sup>

*Effect of Thyroid after Prolonged Cholesterol Feeding*

In view of the similar action of potassium iodide and whole thyroid in preventing a rise in blood cholesterol when given concurrently with cholesterol to rabbits (1), it was of interest to determine also the effect of thyroid upon the blood of these animals with long standing hypercholesterolemia.

TABLE III

*Effect of Whole Thyroid on Long Standing Hypercholesterolemia*

| Rabbit No.   | Blood cholesterol, mg. per 100 cc. |     |     |     |             |     |     |      |
|--------------|------------------------------------|-----|-----|-----|-------------|-----|-----|------|
|              | 10 day periods                     |     |     |     |             |     |     |      |
|              |                                    |     |     |     | Thyroid fed |     |     |      |
| 1-89         | 463                                | 472 | 431 | 278 | 618         | 705 | 862 | 862  |
| 1-93         | 457                                | 581 | 704 | 438 | 506         | 676 | 820 | 736  |
| 1-94         | 452                                | 506 | 486 | 359 | 355         | 589 | 463 | 526  |
| 2-02         | 647                                | 603 | 556 | 263 | 388         | 446 | 495 | 428  |
| 2-06         | 551                                | 376 | 413 | 236 | 284         | 505 | 834 | 1000 |
| 2-23         | 452                                | 481 | 544 | 373 | 556         | 516 | 659 | 833  |
| 2-30         | 422                                | 528 | 464 | 270 | 276         | 305 | 416 | 714  |
| Average..... | 492                                | 507 | 514 | 317 | 426         | 535 | 650 | 729  |

A group of 7 rabbits was used. There were 5 males and 2 females. 4 animals had been fed cholesterol for 19 months and three had received it for 5 months. All had a markedly elevated blood cholesterol. 3 of the rabbits had been used several months previously to determine the effect of potassium iodide upon the hypercholesterolemia.

The cholesterol feeding was continued. Blood samples were taken every 10 days and the cholesterol determined. For a period of 3 weeks, dried whole thyroid was mixed with the grain to which cholesterol had been added. Each rabbit received approximately 0.4 gm. of the thyroid three times a week.

The results are shown in Table III and Fig. 2. The values in the second, third and fourth columns of the table represent a control period of a month before the

<sup>1</sup> The same effect has been observed recently in a 10th rabbit. The blood cholesterol in this animal tripled on potassium iodide, rising from 240 mg. to 821 mg., and dropping back to 213 mg. when the iodide was stopped.

## BLOOD CHOLESTEROL AND ATHEROSCLEROSIS

thyroid was started. In the next two columns are the results obtained during the time that thyroid was fed. The last three columns list the determinations during the month after the thyroid feeding.

Contrary to the effect of potassium iodide, which produced a rise in the level of the hypercholesterolemia, thyroid caused a sharp fall. This decrease, however, was not sustained, and even before the thyroid administration was stopped the blood cholesterol began to rise toward

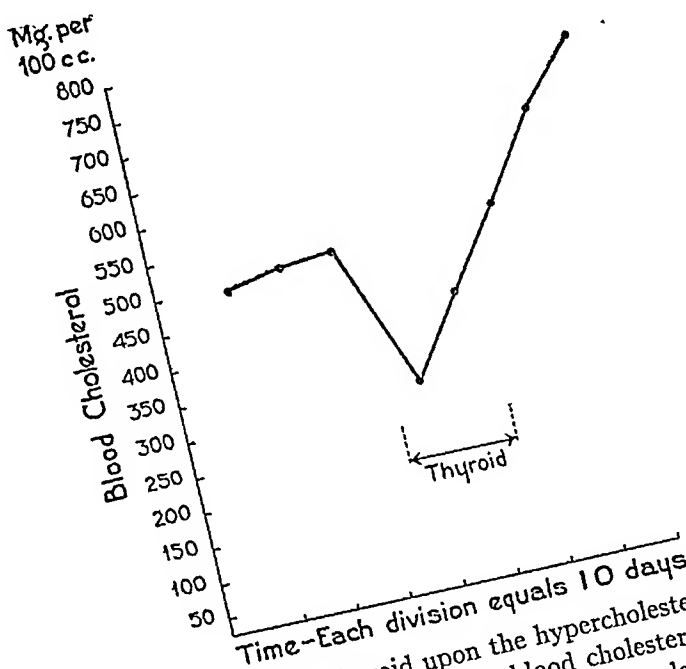


FIG. 2. The effect of whole thyroid upon the hypercholesterolemia of rabbits. After an immediate and marked drop in the blood cholesterol, it begins to rise even before the thyroid is discontinued and finally reaches a level higher than that in the preceding control period.

its previous level. Following the cessation of thyroid feeding the amount of cholesterol in the blood not only reached its former value but actually rose considerably higher than before. This is shown by the curve in Fig. 2.

#### *Relative Effectiveness of Whole Thyroid and Thyroxin*

In previous work (1) thyroxin had seemed somewhat less effective than whole thyroid in preventing a rise in the blood cholesterol of

rabbits fed cholesterol. In an attempt to amplify this finding a group of rabbits was subjected to a complete thyroidectomy. These animals, when fed cholesterol and potassium iodide, should exhibit a prompt rise in blood cholesterol, as the iodide is ineffective in preventing this in the absence of the thyroid (2). However, if whole thyroid or thyroxin were administered in an effort to substitute for the removed gland, the rise in blood cholesterol might be prevented. Furthermore, this procedure could throw some light upon the relative effectiveness of the two substances.

Thyroidectomized rabbits were divided into two groups, each containing 9 animals. Blood cholesterol determinations were made every 10 days. About a month was allowed after the operation to permit the animals to recover completely and to make sure that no spontaneous rise in blood cholesterol was to occur. Both groups were then started on cholesterol and potassium iodide given according to the usual procedure thrice weekly. In addition, each rabbit in the first group received 0.4 gm. of dried whole thyroid mixed with its grain three times a week. Each animal in the second group received 0.7 mg. of thyroxin by hypodermic injection twice a week. Thus the rabbits in the two groups received roughly equivalent dosage so far as thyroxin was concerned.

At the end of the feeding period of 110 days the surviving rabbits were killed. At autopsy the complete removal of the thyroid was verified and the aorta examined in the gross for atheromatous plaques.

The blood cholesterol determinations are given in Tables IV and V. Curves representing the average values are shown in Fig. 3.

The results were unsatisfactory. Examination of the tables reveals that the blood cholesterol of the individual rabbit was subject to considerable fluctuation but that, in most instances, there was a general tendency for it to rise. In other words, with the dosage used, it was impossible wholly to compensate for the removal of the thyroid by giving dried whole thyroid by mouth or thyroxin subcutaneously. It is fair to say, however, that the rise in blood cholesterol was less rapid than might be expected in a comparable group fed cholesterol alone.

Nor did the experiment show any noteworthy difference between the effectiveness of whole thyroid and thyroxin, although the averages of the former group were somewhat lower than the latter, as shown in Fig. 3. This difference is less convincing when it is observed that one of the animals (No. 1-64) in the group receiving thyroxin had a much

## BLOOD CHOLESTEROL AND ATHEROSCLEROSIS

higher blood cholesterol than any of the others in the same group or, of more importance, in the group given whole thyroid. This necessarily resulted in a distortion of the averages.

TABLE IV  
*Thyroidectomized Rabbits Given Cholesterol, Potassium Iodide and Whole Thyroid*

| Thyroidectomized Rabbits Given Cholesterol, 2 |      |     |     |     |     |     |     |     |     |     |     |     |
|---|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Blood cholesterol, mg. per 100 cc.            |      |     |     |     |     |     |     |     |     |     |     |     |
| Rabbit No.                                    | Days |     |     |     |     |     |     |     |     |     |     |     |
|   | 1    | 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 |
| 1-61  | 135  | 90  | 104 | 139 | 121 | 158 | 170 | 138 | 182 | 200 | 200 | 184 |
| 1-79  | 103  | 94  | 134 | 109 | 144 | 188 | 194 | 163 | 164 | 127 | 148 | 229 |
| 2-07  | 132  | 127 | 151 | 175 | 191 | 252 | 197 | 226 | 253 | 284 | 395 | 229 |
| 2-08  | 128  | 116 | 120 | 130 | 211 | 271 | 163 | 276 | 250 | 280 | 291 | 303 |
| 2-09  | 127  | 139 | 182 | 169 | 203 | 242 | 211 | 284 | 308 | 264 | 347 | 347 |
| 2-10  | 124  | 138 | 112 | 133 | 134 | 136 | 184 | 166 | 223 | 162 | 253 | 189 |
| 2-11  | 123  | 112 | 146 | 135 | 185 | 137 | 150 | 159 | 156 | 156 | 268 | 221 |
| 2-12  | 108  | 102 | 123 | 112 | 123 | 129 | 126 | 129 | 107 | 107 | 140 | 108 |
| 2-13  | 108  | 199 | 243 | 241 | 278 | 202 | 166 | 159 | 155 | 114 | 164 | 142 |
| Average..                                     | 121  | 124 | 146 | 149 | 177 | 191 | 173 | 189 | 200 | 188 | 245 | 217 |

TABLE V

Iodide and Thyroxin

TABLE V  
*Thyroidectomized Rabbits Given Cholesterol, Potassium Iodide and Thyroxin*

| Thyroidectomized Rabbits Given Cholesterol |      |     |     |     |     |     |     |     |     |     |     |     |
|--|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Blood cholesterol, mg. per 100 cc.         |      |     |     |     |     |     |     |     |     |     |     |     |
| Rabbit No.                                 | Days |     |     |     |     |     |     |     |     |     |     |     |
|  | 1    | 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 |
| 1-58                                       | 99   | 117 | 122 | 101 | 163 | 187 | 302 | 264 | 210 | 292 | 320 | 250 |
| 1-62                                       | 116  | 104 | 121 | 140 | 112 | 154 | 198 | 237 | 250 | 190 | 208 | 216 |
| 1-64                                       | 104  | 134 | 178 | 228 | 166 | 280 | 318 | 493 | 426 | 646 | 694 | 721 |
| 1-76                                       | 95   | 131 | 96  | 80  | 74  | 98  | 182 | 154 | 88  | 121 | 142 | 160 |
| 2-14                                       | 112  | 167 | 172 | 211 | 218 | 184 | 250 | 232 | —   | —   | —   | —   |
| 2-16                                       | 169  | 149 | 150 | 158 | 155 | 188 | 208 | —   | —   | —   | —   | —   |
| 2-17                                       | 140  | 150 | 175 | 150 | 134 | 159 | 169 | 160 | 272 | 272 | 272 | 253 |
| 2-18                                       | 97   | 145 | 211 | 171 | 215 | 293 | 293 | 280 | 341 | 284 | 347 | 354 |
| 2-19                                       | 110  | 158 | 250 | 153 | 149 | 195 | 232 | 221 | 247 | 223 | —   | —   |
| Average..                                  | 116  | 139 | 169 | 155 | 154 | 193 | 239 | 255 | 262 | 290 | 331 | 326 |

... unsatisfactory in these respects it is generally accepted that

While the experiment was unsatisfactory in these respects it is perhaps important from another aspect. It is generally accepted that

a high blood cholesterol in a rabbit leads to the development of atheromatous plaques in the aorta. Although most of the rabbits in these two groups showed significant rises in blood cholesterol, it is noteworthy that at autopsy gross atherosclerosis of the aorta was present in only 1 animal of each group (Nos. 1-64, 2-09) and in each case the changes were minimal. This corroborates the findings of Page and Bernhard (4) who reported that rabbits given cholesterol and iodide developed a lipemia but that atherosclerosis was slight or absent at autopsy.

This finding becomes all the more perplexing when it is recalled that those rabbits that escaped from the protective action of large doses of

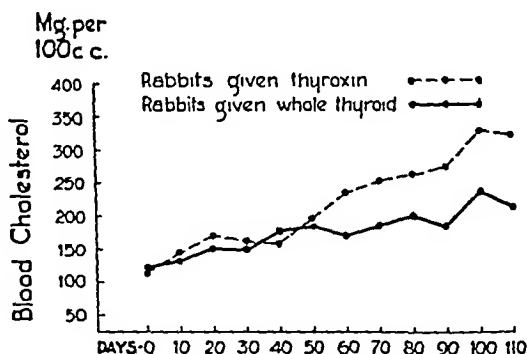


FIG. 3. The comparative effect of thyroid and thyroxin on the blood cholesterol of thyroidectomized rabbits fed cholesterol and potassium iodide.

iodide, as reported above, and developed a hypercholesterolemia all showed atherosclerosis at autopsy. Thus it seems very possible that iodide delays but does not ultimately prevent atherosclerosis in the presence of a high blood cholesterol. Perhaps if the rabbits in the present groups had been fed cholesterol over a longer period they would have shown atherosclerotic changes.

#### *Effect of Age upon Response to Cholesterol Feeding*

Individual rabbits differ markedly in the response of their blood cholesterol to cholesterol feeding. Although a rise usually occurs, this increase varies in degree. Cholesterol is distinctly an abnormal component of an herbivore's diet. In a sense, then, the less a rabbit's blood cholesterol rises when large amounts of cholesterol are added



the diet, the more efficient that animal is in utilizing the mechanism whereby the result is obtained is immaterial.

It seemed possible that an age factor might be present—*i.e.*, the older the rabbit, the less able its organism would be to adjust itself to the presence of an unusual dietary substance and the higher would be the resultant rise in blood cholesterol.

TABLE VI  
Adjustment of Hypercholesterolemia

TABLE VI  
Effect of Age on Development of Hypercholesterolemia  
Blood cholesterol, mg. per 100 cc.

| Rabbit No.    | Blood cholesterol, mg. per 100 cc. |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|---------------|------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|               | Weeks                              |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|               | 0                                  | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  |
| Young Rabbits |                                    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2-51          | 128                                | 135 | 158 | 169 | 187 | 165 | 171 | 247 | 236 | 269 | 192 | 139 | 135 | 138 | 140 | 126 | 138 |
| 2-52          | 130                                | 145 | 200 | 264 | 264 | 305 | 318 | 536 | 454 | 472 | 435 | 222 | 417 | 340 | 259 | 172 | 150 |
| 2-53          | 140                                | 179 | 156 | 174 | 170 | 241 | 234 | 223 | 190 | 191 | 150 | 153 | 120 | 113 | 132 | 118 | 124 |
| 2-54          | 143                                | 172 | 165 | 193 | 189 | 191 | 229 | 307 | 263 | 187 | 236 | 189 | 139 | 159 | 118 | 109 | 92  |
| 2-55          | 142                                | 163 | 142 | 200 | 208 | 210 | 250 | 221 | 179 | 175 | 170 | 151 | 172 | 130 | 125 | 106 | 107 |
| Average..     | 137                                | 137 | 159 | 164 | 200 | 204 | 222 | 240 | 307 | 264 | 259 | 227 | 171 | 197 | 176 | 155 | 126 |
| Old Rabbits   |                                    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2-56          | 137                                | 177 | 189 | 276 | 312 | 469 | 625 | 604 | 602 | 556 | 532 | 476 | 550 | 432 | 428 | 376 | 327 |
| 2-57          | 132                                | 169 | 159 | 213 | 247 | 368 | 536 | 568 | 694 | 650 | 633 | 435 | 318 | 376 | 249 | 207 | 200 |
| 2-58          | 134                                | 158 | 138 | 155 | 188 | 186 | 240 | 264 | 379 | 313 | 261 | 172 | 142 | 156 | 121 | 138 | 118 |
| 2-59          | 137                                | 164 | 130 | 150 | 240 | 318 | 332 | 468 | 340 | 156 | 117 | 113 | 88  | 115 | 122 | 113 | 113 |
| 2-60          | 136                                | 206 | 195 | 293 | 329 | 493 | 536 | 658 | 640 | 618 | 458 | 362 | 316 | 355 | 424 | 379 | 363 |
| Average..     | 135                                | 175 | 162 | 217 | 263 | 367 | 454 | 512 | 531 | 458 | 400 | 312 | 283 | 287 | 269 | 243 | 224 |

To test this thesis, the results of cholesterol feeding upon the blood cholesterol of two groups of rabbits—one young, the other old—was observed. There were 5 animals in each group, and in each case there were 4 females and 1 male. The rabbits in the first group were 4 to 5 months old at the beginning of the experiment; the age of those in the second group ranged from 2 to 3 years. The feeding of cholesterol was begun simultaneously for both groups and was carried out by the usual method.

The results are shown in Table VI and Fig. 4. During the cholesterol feeding the blood cholesterol of the older rabbits rose faster and to much higher levels than that of the young rabbits. When the feeding was stopped the resulting fall in blood cholesterol was more gradual and protracted in the older group. Admittedly the number of rabbits was small and no sweeping conclusions may be drawn. Nevertheless, the difference in response of the two groups was striking.

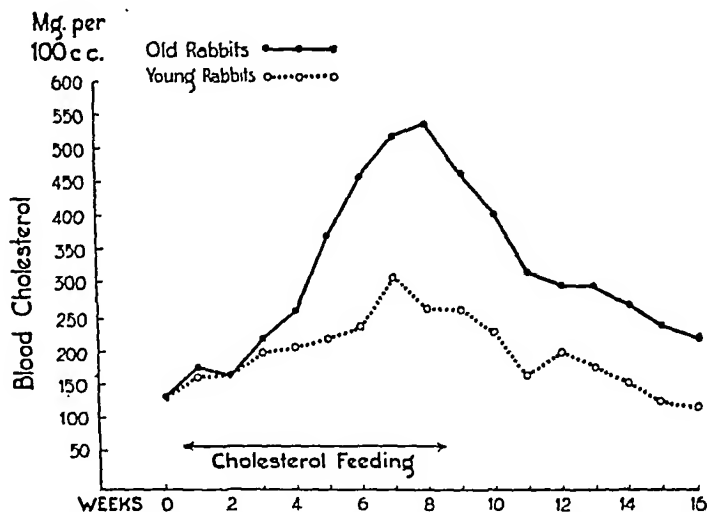


FIG. 4. Curves comparing the results of cholesterol feeding on the blood cholesterol of young and old rabbits. The rise in the latter is more abrupt and more marked; the corresponding fall is more gradual and prolonged.

#### SUMMARY

1. The action of potassium iodide in preventing a significant rise in the blood cholesterol of rabbits fed cholesterol was temporary. After about 4 months it lost its effectiveness and the blood cholesterol rose.

2. In rabbits with hypercholesterolemia resulting from long continued cholesterol feeding, the administration of potassium iodide caused a marked rise in the blood cholesterol.

3. On the other hand, dried whole thyroid given to such animals produced a sharp fall in the blood cholesterol. This fall was temporary and was followed by a rise to new high levels.

4. In thyroidectomized rabbits fed cholesterol and potassium iodide,

both thyroid and thyroxin delayed but did not prevent a rise in blood cholesterol. Even with the hypercholesterolemia in these animals, however, the incidence of atherosclerosis was low.

5. Age apparently played a part in determining the response of the blood cholesterol to cholesterol feeding. In a group of old rabbits when compared with a younger group the rise in the cholesterol of the blood was greater and the subsequent return toward normal was slower when the feeding was stopped.

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# SEROLOGICAL EVIDENCE OF IMMUNITY WITH COEXISTING SENSITIZATION IN A TYPE OF HUMAN ALLERGY (HAY FEVER)\*

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PLATE 31

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Using ragweed hay fever as an example of a type of allergy we record serological studies which were undertaken to explain the protection resulting from pollen injections. The results have indicated to us the production of an inhibiting or immune type of substance that prevented the allergen from reacting with the sensitized cell, and they have also demonstrated the coexistence of both sensitizing and immune antibodies in the specifically treated patients.

The term allergy is used today in a general sense to designate a number of specific reactions that have important clinical, pathological and immunological differences.

It seems possible now to differentiate one group occurring spontaneously in man; that is, without artificial parenteral stimulation, subject to hereditary influence and evidenced clinically by an edematous type of reaction that quickly follows contact of the allergen with the sensitized cell. Immunologically this group is characterized by skin and mucous membrane sensitization with the sensitizing antibody demonstrable in the blood serum as well as by the entire absence from the blood of precipitins and smooth muscle sensitizing antibody so regularly found in artificially induced allergy (anaphylaxis). A review of the literature on these points together with additional studies is contained in an article by Cooke and Spain (1). De Besche (2) has confirmed this work.

With the discovery of the transferable skin sensitizing antibodies in the serum

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of allergies of this group, Prausnitz and Küstner (3) explained, in part at least, the mechanism of this type of allergy.

The pollen sensitization of man (hay fever) is representative of this group. The beneficial result of specific therapy in pollen allergy has rested upon clinical observation. As a result of many independent studies for more than 20 years, it is now accepted as a fact that such injections afford satisfactory clinical immunity, but there has not yet been offered any solution of the protective mechanism. On account of its frequency, ragweed hay fever afforded the best opportunity for such a study.

#### EXPERIMENTAL

On the theory that it might be possible to demonstrate the existence of an immune substance in the blood of treated hay fever cases, we first proceeded to note the clinical effects of transfusion, using treated patients as blood donors for untreated patients with the same sensitization having active hay fever, some with asthma, in the beginning of the pollen season.

In 1931, 1932, 1933 and 1934 twenty cases were transfused and the effects carefully noted. The donors used had all been actively and continuously treated for at least a year, but in no case had a donor received a pollen injection within 2 weeks of the transfusion. At the time of transfusion all donors were positive by intradermal test to ragweed pollen extract and their serum transferred the sensitization to normal human skin. The donors themselves had satisfactory clinical results through the balance of the pollen season.

The recipients of this theoretically immune blood received no treatment other than transfusion and lived their usual lives in the usual pollen atmosphere. Sixteen of the twenty cases had satisfactory results which lasted through the remaining 4 to 5 weeks of their hay fever season. 96 other patients received repeated 10 cc. doses of the supposedly immune serum subcutaneously with clinical improvement in 60 per cent.

These results, in some cases striking, indicated to us the presence of a transferable protecting substance which we then sought to demonstrate by serological studies.

In this work the method of passive transfer was employed, using the skin of normal non-allergic people as test subjects to provide test sites made with serum of patients sensitive clinically and by test to ragweed pollen. Bleedings were made and serum obtained from the patient group before and after treatment with ragweed extract. Hereafter in this paper the term test subject denotes the non-

sensitive group whose skin was used for sites and tests. The ante-treatment serum is called Serum A, and the post-treatment serum called Serum P. The pollen extracts used throughout this work were prepared from ethyl ether extracted pollen in alkaline saline fluid and were standardized on the protein nitrogen basis (4) (100 units=0.001 mg. protein nitrogen). Dilutions were made in physiologic salt solution. As near as we can estimate 60,000 of our units equalled 100,000 Noon units (5) calculated on a weight by volume basis as used by Harley (5).

The synopsis of the histories and essential facts regarding the eight cases whose serums in special were studied for this paper is as follows:

St. Case 2025. Male. Age 40. Ragweed hay fever with asthma of 12 years duration. Some ragweed injections in 1929. Intradermal test with ragweed 1,000 units marked (+ + + +). Tests after treatment were approximately the same. Serum A taken Aug. 10, 1933. He was transfused on Aug. 15, 1933, and was one of the sixteen cases referred to with satisfactory results in 1933. Treated at weekly intervals with giant ragweed from Oct. 24, 1933, with moderate constitutional reactions when dosage exceeded 10,000 units. Dose reduced to 5,000 units. The last injection was given Oct. 2, 1934. Serum P taken Oct. 24, 1934, when total dosage had been 230,000 units. 90 per cent relief in 1934 (3 days of hay fever).

Sc. Case 2105. Male. Age 46. Ragweed hay fever since 1931. Never previously treated. Intradermal test with ragweed 1,000 units marked (+ + + +). Test after treatment showed very slight reduction in activity. Serum A taken Sept. 6, 1933. Treated at weekly intervals with giant ragweed until 10,000 units had been reached, when injections were given every 2 weeks and finally every month. Last five injections 60,000 units each with dosage totalling 585,535 units. Last dose given Nov. 17, 1934. Serum P was taken Jan. 18, 1935. 80 per cent relief (7 days hay fever).

Ce. Case 2055. Female. Age 31. Ragweed hay fever since 1932. Never previously treated. Intradermal test with ragweed 1,000 units marked (+ + + +) before treatment. This was reduced during treatment, and was only slight (+) to 1,000 units after treatment. Serum A taken Oct. 20, 1933. Treated at weekly intervals with giant ragweed until 60,000 units was reached. Last injection Aug. 8, 1934, dosage totalled 301,700 units. Serum P taken Sept. 15, 1934. 90 per cent relief (3 days hay fever).

Bu. Case 2022. Male. Age 55. Ragweed hay fever of 15 years duration. Never previously treated. Intradermal test with ragweed marked (+ + + +) to 1,000 units before treatment and approximately the same after treatment. Serum A taken Nov. 6, 1933. Treatment started at weekly intervals with giant ragweed until 60,000 units was reached on Sept. 7, 1934. Total dosage 430,480 units. Serum P taken Oct. 1, 1934. Had no hay fever in 1934.

Kr. Case 2042. Male. Age 10. First definite hay fever in 1933 was untreated. Intradermal test before treatment on Nov. 10, 1933, marked (+ + + +) to 1,000

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units and the same after treatment. Serum A taken Nov. 13, 1933. Treated with low ragweed at weekly intervals until receiving 10,000 units in June, 1934. Treatment interrupted until September, 1934, when dosage was reduced to 5,000 units. Last dose Sept. 25, 1934, 5,000 units, totalling 93,190 units. Serum P taken Nov. 2, 1934. 70 per cent relief (9 days of hay fever).

Bl. Case 2076. Male. Age 11. Ragweed hay fever since 1932. Never previously treated. Intradermal test marked (++++) to 1,000 units ragweed before treatment. Tests after treatment showed slight reduction of activity. Serum A taken Sept. 6, 1933. Treated with low ragweed at weekly intervals until 21,000 units was reached and a constitutional reaction resulted. Dosage was reduced, last injection Oct. 15, 1934, 5,000 units, totalling 192,280 units. Serum P taken Nov. 7, 1934. 70 per cent relief (9 days of hay fever).

Sp. Case 2054. Male. Age 47. Ragweed hay fever since 1932. Never previously treated. Transfused with satisfactory results on Sept. 7, 1933. Intradermal reaction marked (++++) to 1,000 units ragweed before treatment. Reactions after treatment were a trifle less in activity. Serum A taken Sept. 19, 1933. Treated at weekly intervals from October, 1933, with giant ragweed up to 10,000 units when the interval was increased to 2 weeks and dosage increased to 60,000 units. Total units 473,650. Last injection Sept. 21, 1934. Serum P taken Oct. 5, 1934. 70 per cent relief (9 days hay fever).

Bo. Case 2045. Female. Age 38. Ragweed hay fever since 1931. Never previously treated. Intradermal reactions reduced to moderate (++) to 100 units ragweed. After treatment skin reactions reduced to moderate (++) to 100 units. Serum A taken Aug. 30, 1933. Transfused on Aug. 31 with excellent results in 1933. Treatment started Oct. 20, 1933, with giant ragweed until 60,000 unit dose was given. Kept at that level for four doses until Sept. 10, 1934, the date of last injection. Dosage totalled 519,240 units. Serum P taken Sept. 20, 1934. 85 per cent relief in 1934 (5 days of hay fever).

Precipitin tests were done on both Serum A and Serum P of all these cases except Kr. and Bo. The antigens used were giant and low ragweed extracts and their pure proteins. There was no precipitation.

Our observations consisted of a comparison of the Serum A and Serum P of each of these eight cases with reference to:

1. The relative amounts of skin sensitizing antibody in the Serums A and P to determine its increase, decrease or disappearance under treatment.
2. The reactions of test subjects' skin both to injections of serum-allergen mixtures at the time the transfer sites were made and to the subsequent tests of these sites.

The normal skin test subjects vary somewhat both as to their acceptance of transfer and their reactivity to test, hence crucial comparisons must be made on

the same test subject at the same time. Extracts of pollen deteriorate. We have found this to be definite and demonstrable in extracts 2 months old but not at 1 month. The extracts used in this work were made fresh every month. The serums must be kept sterile. The question arose early as to the possible loss of skin sensitizing capacity of aging serum. Levine and Coca (6) have stated there was no apparent loss in 3 months' time. Our tests indicated none in 1 year when serum was kept sterile at 8°C. The non-specific reaction produced by heterologous serums when injected intradermally for skin sites has introduced some difficulty. Chant and Gay (7) studied this point and stated that such reactions started at once, reached a maximum in 15 minutes and began to fade in less than 30 minutes. Our observations were in entire accord. These "irritative" reactions will be discussed later under serum-allergen mixture tests as they have been a cause of erroneous interpretation by previous observers.

### *1. Titration of the Relative Amounts of Skin Sensitizing Antibody in Serums before and after Treatment*

Influenced by observations in experimental anaphylaxis the effects of pollen therapy are generally ascribed to desensitization. In the type of allergy represented by hay fever cellular antibody may be tested for through the skin by prick, scratch or intradermal method. Using the intradermal method, Cooke (8), Levine and Coca (9) and others have recorded no material objective change in the test in the majority of cases treated to a successful clinical result. Markin (10), Brown (11) and Harley (12) on the contrary have reported abolition of skin reaction after treatment using the scratch or prick method. These discrepancies may be due to the difference of technique and may be explainable, but if one claims cellular desensitization it should be only after use of the more delicate test, the intradermal, and not the less delicate, the scratch or prick.

In the summaries of the eight cases here presented, no significant differences could be noted in the intradermal reactions before and after treatment with the exception of Ce., No. 2055, where it was definitely reduced but not abolished. Cellular desensitization was not demonstrable in these long and excessively treated cases using the intradermal test.

The amount of skin sensitizing antibody in the serum has been determined by injecting increasing dilutions of sensitive serum into normal skin (passive transfer) and testing these sites 24-48 hours later. Levine and Coca (6) found no decrease of serum antibody in the serum



**TABLE I**  
*Titration of the Amount of Skin Sensitizing Antibody in Serums before and after Treatment*

| Serum dilutions* | Bu. serum†          |         |                     |         | Kr. serum†          |         |                     |         |
|------------------|---------------------|---------|---------------------|---------|---------------------|---------|---------------------|---------|
|                  | (Test subject D.L.) |         | (Test subject M.G.) |         | (Test subject H.R.) |         | (Test subject F.S.) |         |
|                  | Serum A             | Serum P | Serum A             | Serum P | Serum A             | Serum P | Serum A             | Serum P |
|                  |                     |         |                     |         |                     |         |                     |         |
| 1-10             | +++                 | ++      |                     |         | ++++                | ++++    | ++++                | ++++    |
| 1-25             |                     |         |                     |         | +++                 | +++     | ++++                | ++++    |
| 1-50             |                     |         |                     |         | +++                 | ++      | ++++                | +++     |
| 1-100            | ++                  | ++      |                     |         | +++                 | +       | +++                 | +++     |
| 1-200            | ++                  | +       | ++                  | +       |                     |         | ++                  | ++      |
| 1-300            | +                   | 0       | +                   | ±       | +                   | +       | +                   | +       |
| 1-400            | ±                   | 0       | ±                   | 0       |                     |         |                     |         |
| 1-500            | ±                   | 0       |                     |         |                     |         |                     |         |
| 1-700            |                     |         |                     |         |                     |         |                     |         |

| Serum dilutions* | Bo. serum†          |         | St. serum†          |         | Ce. serum†          |         | Sc. serum†          |         |
|------------------|---------------------|---------|---------------------|---------|---------------------|---------|---------------------|---------|
|                  | (Test subject E.S.) |         | (Test subject G.W.) |         | (Test subject J.N.) |         | (Test subject O.C.) |         |
|                  | Serum A             | Serum P | Serum A             | Serum P | Serum A             | Serum P | Serum A             | Serum P |
|                  |                     |         |                     |         |                     |         |                     |         |
| 1-10             | +++                 | ++++    | +++                 | +++     | ++                  | ++      | ++                  | ++      |
| 1-25             | ++                  | +++     | +++                 | +++     | ++                  | +       | ++                  | ++      |
| 1-50             | +                   | ++      | +++                 | +++     | 0                   | ±       | +                   | +       |
| 1-100            | ±                   | +       | +++                 | +++     | 0                   | 0       | +                   | +       |
| 1-200            | 0                   | ±       | +                   | +       |                     |         |                     |         |
| 1-300            |                     |         |                     |         |                     |         |                     |         |
| 1-400            |                     |         | ±                   | +       |                     |         |                     |         |
| 1-500            |                     |         |                     |         |                     |         |                     |         |

| Serum dilutions* | Sp. serum†          |         |                     |         | Bl. serum†          |         |                     |         |
|------------------|---------------------|---------|---------------------|---------|---------------------|---------|---------------------|---------|
|                  | (Test subject G.W.) |         | (Test subject C.T.) |         | (Test subject C.T.) |         | (Test subject H.R.) |         |
|                  | Serum A             | Serum P | Serum A             | Serum P | Serum A             | Serum P | Serum A             | Serum P |
|                  |                     |         |                     |         |                     |         |                     |         |
| 1-10             | ++++                | ++++    | ++++                | ++++    | ++++                | ++++    | +++                 | +++     |
| 1-25             | +                   | +++     |                     |         |                     |         | ++                  | +++     |
| 1-50             | +                   | +++     | ±                   | ++++    | +                   | +++     | ±                   | ++      |
| 1-100            | ±                   | +++     | 0                   | +++     | ±                   | +       | 0                   | ++      |
| 1-200            |                     |         | 0                   | ++      | 0                   | 0       | 0                   | +       |
| 1-300            | 0                   | +++     | 0                   | +       | 0                   | 0       | 0                   |         |
| 1-400            |                     |         | 0                   | ±       | 0                   | 0       |                     |         |
| 1-500            | 0                   | +++     | 0                   |         |                     |         |                     |         |

+ = degree of skin reaction.  
± = doubtful skin reaction.  
0 = negative skin reaction.

A = serum taken before treatment with ragweed extract.

P = serum taken after treatment with ragweed extract.

\* 1/10 cc. of these serum dilutions in physiologic saline was placed in each site.

† The serum dilution sites were tested with 1/40 cc. of low ragweed 100 units per cc. 48 hours after they were made.

of treated patients. In some cases it was materially increased. Gay and Chant's (13) findings were in accord. Harley (12) using the same technique stated, "In one case the serum reagin disappeared completely, in the others they were markedly reduced." Markin's (10) reports agree.

This titration of skin sensitizing capacity of serums is of importance in the interpretation of the results of the mixture experiments later described. One can reasonably assume that a serum with a greater concentration of antibody would transfer its sensitiveness in a higher dilution than a serum in which the antibody was less abundant. If this is so it is also indicated that a serum containing a greater amount of antibody would require a greater amount of allergen to effect desensitization. This is confirmed by our studies, not given here in detail, which have shown us that ante-treatment serums that did not transfer beyond a 1-10 dilution were neutralized by an equal amount of pollen extract containing about 50 units per cc., whereas a serum that transferred in a 1-1,000 dilution required an equal amount of extract containing nearly six times as many units, hence this dilution method is a relatively accurate means of determining the amount of the skin sensitizing antibody.

In the serums of our eight cases shown in Table I the comparison of Serum A with Serum P showed equality of sensitizing antibody for Kr., St., Sc. and Ce. The Serum P antibody was slightly decreased for Bu., slightly increased for Bo. and Bl. and decidedly increased for Sp. As shown in the case histories all these patients received about as much treatment as Harley's cases. Our findings support those of Levine and Coca (6) and Gay and Chant (13) and disagree with those of Markin (10) and of Harley (12).

Serum desensitization was not obtained by us and post-treatment serum in all cases was demonstrably an actively sensitizing serum as shown by passive transfer tests, therefore as normally circulating in the patient's body it must keep the tissues sensitive. That this is true was shown by the direct positive intradermal test on these patients after complete treatment. The only interpretation that can be placed on these findings is that there is no evidence of protection through desensitization, cellular or humoral, nor yet any evidence that the clinical immunity from specific injections might be afforded by an increase of sensitizing antibody.

## 2. *The Reactions of Serum-Allergen Mixtures in Non-Sensitive Test Subjects*

Injections of serum-allergen mixtures into normal skin have been recorded, but no account seemingly has been taken as to whether the serums in these mixtures were obtained before or after treatment. There are no observations in the literature in which a study has been made of the comparative behavior of mixtures of allergen with Serum A and with Serum P such as we now record. Coca and Grove (14), Levine and Coca (6, 15), Clarke and Gallagher (16), Baldwin (17) and Gay and Chant (13) first reported the finding of positive specific reactions. They have been supported by Foran and Lichtenstein (18) and Harley (5), and by our own findings previously (19) and now recorded. These contradictory reports may be due to the failure to discriminate between serum taken before and that taken during or after treatment, or they may be explained by the fact already mentioned that injection of heterologous serum into normal skin produced an irritative non-specific reaction with wheal and erythema readily confused with the specific reaction.

The non-specific reaction begins at once, reaches its maximum in 15 minutes, and with most serums the erythema begins to disappear before 30 minutes and is practically gone in 1 hour, leaving a pale round elevated papule. The specific reaction begins more slowly, and erythema and wheal are still active at the end of an hour at which time these tests should be read.

In making allergen-serum mixtures precautions for sterility were used. A definite amount of a serum was mixed with an equal amount of the allergen in varying strengths, in these cases ragweed pollen extract. The mixtures were allowed to stand about 15 hours. Harley (5) contends that there is a binding of allergen to antibody because of the negative skin reaction in sensitive patients tested with incubated serum-allergen mixtures. Our previous experiments which we shall not give here in detail were carried out in duplicate, one set at 8°C. and the other at 37°C. Care was taken to assure an excess of antibody over allergen. Intradermal tests on ragweed sensitive patients were made with the two mixtures in varying concentrations of extract and

controlled by the usual saline extracts of the same strength. No differences could be observed, hence Harley's assumption of inactivation through binding of antigen by antibody could not be confirmed. The serum mixtures used in this study were kept at 8°C. In order to make this presentation clear the complete protocol of the typical Experiment 26 will be given.

*Experiment 26.*—Bu.'s Serum A and Serum P were each mixed in test tubes with an equal volume of a low ragweed extract (LR34E) of a certain unit strength as indicated in Table II. Also dilutions with physiological saline were made of both Serums A and P. These preparations made Apr. 11, 1935, were allowed to stand at 8°C. overnight and 1/10 cc. of each mixture or dilution was injected intradermally on Apr. 12 into each site in the skin of the back of normal test subject (D. L.). Twenty-two separate sites were made and marked with an indelible pencil. The 1 hour reactions of the ten mixtures were recorded. There was of course no 1 hour reaction with the serum dilutions. The test subject returned on the 14th of April for the tests of the sites. At this time there was injected into each site 1/40 cc. of a ragweed extract (LR34E). We had chosen quite arbitrarily a reasonably strong extract containing 1,000 units per cc. to test mixture sites, and a weaker extract, 100 units per cc., for testing dilution sites. The details of the mixtures and the results are recorded in Table II, and illustrated in Figs. 1 and 2.

These results showed that at the time the sites were made on Apr. 12 the mixtures of ragweed and Serum A gave positive 1 hour reactions but the saline control was negative. (Fig. 1, Column 2.) On the contrary the Serum P ragweed mixtures gave no 1 hour reaction except for a suggestive or doubtful reaction with the 1,000 unit extract. This is significant as it seems to indicate that excess of allergen may jump the immune barrier, so to speak, and this suggests the possibility of measuring the amount of protective substance (Fig. 1, Column 3).

When these ten mixture sites were tested on Apr. 14 the Serum A mixtures that had previously reacted were now negative, whereas the Serum P mixtures previously negative or doubtful were now positive.

The comparison of the saline dilutions of Serum A and Serum P showed a slight but definitely greater amount of sensitizing antibody in Serum A. Considering the extent of treatment recorded in the synopsis of the Bu. case this cannot be interpreted as desensitization sufficient to explain the almost perfect clinical result recorded for that season.

Similar experiments with the serums of the eight cases studied have been grouped and the results recorded in Table III. They are quite strikingly uniform throughout. It is interesting to note that all Serums A (except B1.) had been neutralized by an equal amount of

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TABLE II  
Experiment 26, Test Subject D.L.

| Sites | Apr. 11                |  | Apr. 12   | Apr. 14   |
|-------|------------------------|--|---|---|
|       | Serum-ragweed mixtures |  |   |   |
|       | Equal amounts          | 1 hr. reactions when sites were made, Fig. 1 | Reaction to test with ragweed 1,000 units per cc., Fig. 2 |   |
|       | Bu. serum              | Ragweed extract units per cc.                | (Column 2)  | (Column 2)  |
| 1     | A                      | 50   | +++   | 0   |
| 2     | A                      | 100  | +++   | 0   |
| 3     | A                      | 150  | +++   | 0   |
| 4     | A                      | Saline (control)                             | 0   | ++++  |
| 5     | P                      | 150  | (Column 3)  | (Column 3)  |
| 6     | P                      | 300  | 0   | ++  |
| 7     | P                      | 500  | 0   | ++  |
| 8     | P                      | 700  | 0   | ++  |
| 9     | P                      | 1,000  | ±   | +   |
| 10    | P                      | Saline (control)                             | 0   | +++   |
| Sites | Serum dilutions        |  |   |   |
|       | Bu. serum              | Saline dilution                              | 1 hr. reactions when sites were made, Fig. 1              | Reaction to test with ragweed 100 units per cc., Fig. 2 |
|       |                        |  | (Column 1)  | (Column 1)  |
| 11    | A                      | 1-10   | 0   | +++   |
| 12    | A                      | 1-100  | 0   | ++  |
| 13    | A                      | 1-200  | 0   | ++  |
| 14    | A                      | 1-300  | 0   | +   |
| 15    | A                      | 1-400  | 0   | ±   |
| 16    | A                      | 1-500  | 0   | ±   |
| 17    | P                      | 1-10   | (Column 4)  | (Column 4)  |
| 18    | P                      | 1-100  | 0   | ++  |
| 19    | P                      | 1-200  | 0   | ++  |
| 20    | P                      | 1-300  | 0   | +   |
| 21    | P                      | 1-400  | 0   | 0   |
| 22    | P                      | 1-500  | 0   | 0   |

ragweed 150 units per cc. or less on at least one of two test subjects. This means that the sensitizing antibody had been used up in the reaction at the time the mixtures were injected, and is proved by the

TABLE III

*Reactions at the Time the Mixture Sites Were Tested with Ragweed 48 Hours after They Were Made*

| Mixture for sites* |        |                       | Reaction of sites when tested 48 hrs. later with ragweed† |                      | Mixture for sites* |        |                       | Reaction of sites when tested 48 hrs. later with ragweed† |                     |
|--------------------|--------|-----------------------|---|----------------------|--------------------|--------|-----------------------|---|---------------------|
| Serum              |        | With LR units per cc. |   |                      | Serum              |        | With LR units per cc. |   |                     |
|                    |        |                       | (Test subject M.R.)                                       | (Test subject D. L.) |                    |        |                       | (Test subject M.L.)                                       | (Test subject O.C.) |
| Bu.                | A      | 50                    | +   | 0                    | Sc.                | A      | 50                    | ±   | +                   |
|                    | A      | 100                   | ±   | 0                    |                    | A      | 100                   | 0   | +                   |
|                    | A      | 150                   | ±   | 0                    |                    | A      | 150                   | 0   |                     |
|                    | A      | Saline                | ++++  | ++++                 |                    | A      | Saline                | ++++  | ++++                |
|                    | P      | 150                   | +++   | ++                   |                    | P      | 50                    | ++++  |                     |
|                    | P      | 300                   | +   | ++                   |                    | P      | 100                   | +++   | +++                 |
|                    | P      | 500                   | +   | ++                   |                    | P      | 150                   | +++   | +++                 |
|                    | P      | 700                   | +   | ++                   |                    | P      | 300                   |   | +++                 |
|                    | P      | 1,000                 | ±   | +                    |                    | P      | 700                   |   | ++                  |
| P                  | Saline | ++++                  | +++   | P                    | 1,000              | ±      | ++                    |   |                     |
|                    |        |                       |   |                      | P                  | Saline | ++++                  | ++++  |                     |
| St.                |        |                       | (Test subject M.G.)                                       | (Test subject T.Y.)  | Bl.                |        |                       | (Test subject D.S.)                                       | (Test subject L.L.) |
|                    | A      | 50                    | +   |                      |                    | A      | 50                    | ++  |                     |
|                    | A      | 100                   | +   |                      |                    | A      | 100                   | +   |                     |
|                    | A      | 150                   | 0   | ++                   |                    | A      | 150                   | +   |                     |
|                    | A      | Saline                | +++   | ++++                 |                    | A      | 300                   |   | ±                   |
|                    | P      | 50                    | ++++  |                      |                    | A      | Saline                | ++++  | +++                 |
|                    | P      | 100                   | ++++  |                      |                    | P      | 50                    | ++++  | +++                 |
|                    | P      | 150                   | +++   | +++                  |                    | P      | 100                   | ++++  |                     |
|                    | P      | 300                   |   | +++                  |                    | P      | 150                   | ++++  |                     |
|                    | P      | 500                   |   | +++                  |                    | P      | 300                   |   | ++++                |
|                    | P      | 700                   |   | +                    |                    | P      | 500                   |   | ++++                |
|                    | P      | 1,000                 | +   | +                    |                    | P      | 700                   |   | +++                 |
|                    | P      | Saline                | ++++  | ++++                 |                    | P      | 1,000                 |   | +                   |
|                    |        |                       |   |                      |                    | P      | Saline                | ++++  | ++++                |

+ = degree of skin reaction.

± = doubtful skin reaction.

0 = negative skin reaction.

\* 1/10 of these mixtures of equal volumes of serum and ragweed extract or serum and saline was placed in each site.

† Tested with 1/40 cc. low ragweed 1,000 units per cc.



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TABLE III—*Concluded*

| Mixture for sites* |                       | Reaction of sites when tested 48 hrs. later with ragweed† |                     | Mixture for sites* |                       | Reaction of sites when tested 48 hrs. later with ragweed† |                     |
|--------------------|-----------------------|---|---------------------|--------------------|-----------------------|---|---------------------|
| Serum              | With LR units per cc. | (Test subject N.P.)                                       | (Test subject T.Y.) | Serum              | With LR units per cc. | (Test subject R.H.)                                       | (Test subject L.L.) |
| Kr.                | A                     | 50  | +++                 | Sp.                | A                     | 50  | +                   |
|                    | A                     | 100   | ±                   |                    | A                     | 100   | 0                   |
|                    | A                     | 150   | 0                   |                    | A                     | 150   | 0                   |
|                    | A                     | Saline  | ++++                |                    | A                     | Saline  | +++                 |
|                    | P                     | 50  | ++++                |                    | P                     | 50  | ++++                |
|                    | P                     | 100   | ++++                |                    | P                     | 100   | ++++                |
|                    | P                     | 150   | ++++                |                    | P                     | 150   | ++++                |
|                    | P                     | 300   | ++                  |                    | P                     | 300   | +++                 |
|                    | P                     | 500   | ++                  |                    | P                     | 500   | +++                 |
|                    | P                     | 700   | 0                   |                    | P                     | 700   | +++                 |
|                    | P                     | 1,000   | ++++                |                    | P                     | 1,000   | ++++                |
|                    | P                     | Saline  | ++++                |                    | P                     | Saline  | ++++                |
|                    |                       |   |                     |                    |                       |   |                     |
|                    |                       |   |                     |                    |                       |   |                     |
| Ce.                | A                     | 50  | 0                   | Bo.‡               | A                     | 50  | +                   |
|                    | A                     | 100   | 0                   |                    | A                     | 100   | 0                   |
|                    | A                     | 150   | 0                   |                    | A                     | 150   | 0                   |
|                    | A                     | Saline  | ++++                |                    | A                     | Saline  | ++++                |
|                    | P                     | 50  | +++                 |                    | P                     | 50  | ++++                |
|                    | P                     | 100   | +++                 |                    | P                     | 100   | ++++                |
|                    | P                     | 150   | +                   |                    | P                     | 150   | ++++                |
|                    | P                     | 300   | ++                  |                    | P                     | 1,000   | ±                   |
|                    | P                     | 500   | ++                  |                    | P                     | Saline  | ++++                |
|                    | P                     | 700   | ++                  |                    |                       |   |                     |
|                    | P                     | 1,000   | 0                   |                    |                       |   |                     |
|                    | P                     | Saline  | ++++                |                    |                       |   |                     |
|                    |                       |   |                     |                    |                       |   |                     |
|                    |                       |   |                     |                    |                       |   |                     |

‡ To the poorly transferring Bo. serum treated and untreated a small amount of Fe.'s untreated serum was added to increase its skin sensitizing capacity.

fact that the 48 hour tests of the sites were negative. In striking contrast is the fact that when Serum P (except Kr.) was used in the mixtures, every site was still giving positive reaction when tested 48 hours later, although a much greater amount of ragweed (1,000 units per cc.) had been used in the mixtures. In other words, Serum P had not been neutralized by a reaction when sites were made even with

this strong extract. Since we have already shown that there was no greater amount of sensitizing antibody (except Sp.) to require this additional antigen, we feel justified in assuming the presence of an inhibiting agent in the serum of patients after they had been specifically treated.

Before proceeding to our interpretation of these findings we shall record a few supplementary experiments that aid in the solution.

It was necessary to know that an inhibiting Serum P would exert the effect on the actively sensitizing Serum A of another patient. This was done by combining Bo. Serum P with Fe. Serum A controlled by Bo. Serum A and Fe. Serum A. The inhibiting effect was demonstrable (see Table III).

It was then shown that normal non-sensitive human serum combined with Fe. Serum A did not have an inhibiting effect.

The next point was to discover whether Serum P had any binding, inactivating or lytic effect on the allergen. It has already been noted that there was no discoverable precipitin in the six serums studied for precipitins. If Serum P-ragweed mixtures would give reactions equal to Serum A-ragweed mixtures when tested on sensitive cases, then binding or destruction of allergen could not be maintained. The following experiment was done.

To Bu. Serum A was added an equal volume of ragweed extract 700 units per cc. A similar mixture was made with Bu. Serum P, and Bu. Serum P with saline was used as control. These three mixtures were incubated at 37°C. for 12 hours and then were diluted with saline so that there were, of each mixture, three dilutions containing 1, 5 and 10 units of ragweed per cc. respectively. These nine dilutions were then tested on ragweed sensitive cases. The results shown in Table IV indicated no lessening in the activity of the allergen in the Serum P mixture, hence no allergen destruction.

Another question of importance concerned the specificity of the inhibiting substance. The experiments already recorded, as for example the Serum P of Bu. who was treated with giant ragweed, and Serum P of Bl. who was treated with low ragweed, had both shown the inhibiting effect against the low ragweed extract (Table III). Experiments which we will not give in detail have further shown that Serum P from these two cases also showed an inhibition of the reaction against giant ragweed. Other cases treated with low ragweed extract

showed the inhibition of reaction by Serum P against giant as well as low ragweed extract, and serum of cases treated with giant extract inhibited both giant and low ragweed extract. Thus both species of ragweed produced a common inhibiting substance, and this supports

TABLE IV  
*Immediate Skin Reactions on Ragweed Sensitive Cases*

| Ragweed sensitive cases | <i>Mixture 1*</i><br>Bu. Serum A and low ragweed |      |      | <i>Mixture 2†</i><br>Bu. Serum P and low ragweed |      |      | <i>Mixture 3‡</i><br>Bu. Serum P and saline (control) in similar dilutions to preceding mixtures |   |   |
|-------------------------|--|------|------|--|------|------|--|---|---|
|                         | Units per cc.                                    |      |      | Units per cc.                                    |      |      | +  | 0 | + |
|                         | 1  | 5    | 10   | 1  | 5    | 10   |  |   |   |
|                         | 1  | 5    | 10   | 1  | 5    | 10   |  |   |   |
| 1                       | +++  | ++++ | ++++ | +++  | ++++ | ++++ | +  | 0 | + |
| 2                       | +  | ++   | +++  | +  | +    | +    | 0  | + | + |
| 3                       | ++   | +++  | +++  | ++   | +++  | +++  | ±  | 0 | ± |
| 4                       | 0  | 0    | +    | +  | +    | +    | 0  | 0 | 0 |
| 5                       | +  | ++   | +++  | +  | ++   | +++  | 0  | 0 | 0 |
| 6                       | +  | +    | +++  | ++   | +++  | +++  | 0  | 0 | ± |
| 7                       | ++   | +++  | +++  | ++   | +++  | +++  | ±  | 0 | 0 |
| 8                       | ++   | +++  | +++  | ++   | +++  | +++  | 0  | 0 | 0 |
| 9                       | ++   | +++  | +++  | +  | +++  | +++  | 0  | 0 | ± |
| 10                      | +  | +++  | +++  | ++   | ++   | +++  | ±  | ± | ± |
| 11                      | ++   | +++  | +++  | ++   | +++  | +++  |  |   |   |
| 12                      | ++   | +++  | +++  | +++  | +++  | +++  |  |   |   |

+ = extent of skin reaction.  
± = doubtful skin reaction.  
0 = negative skin reaction.

\* *Mixture 1*.—Bu. Serum A (taken before ragweed treatment) and low ragweed 700 units in equal parts incubated at 37°C. for 12 hours, then diluted with buffered saline to 1, 5, 10 units of ragweed per cc.

† *Mixture 2*.—Bu. Serum P (taken after ragweed treatment) and low ragweed 700 units in equal parts incubated at 37°C. for 12 hours, then diluted with buffered saline to 1, 5, 10 units of ragweed per cc.

‡ *Mixture 3 (Control)*.—Bu. Serum P and saline in equal parts incubated at 37°C. for 12 hours, then diluted comparable to 1, 5 and 10 units of Mixtures 1 and 2.

our clinical experience and our previous conclusion (19) that as allergens they are qualitatively alike.

More important still is the question of whether the inhibiting substance created by the injection of ragweed pollen extract in the

specifically sensitive subject inhibited the skin reaction of timothy pollen mixed with its specific serum.  
The following experiment was done.

Serums from cases of timothy hay fever sensitive only to timothy pollen, taken before any treatment with timothy extract, were mixed with ragweed sensitive Serum A and ragweed sensitive Serum P. Equal volumes of the serum combinations with timothy extract or serum combinations with saline (control) were mixed and placed at 8° C. for 18 hours. Sites were made with 1/10 cc. of these

TABLE V  
*Specificity of the Ragweed Inhibiting Substance*

| TABLE V   |   |   |   |   |   |   |                       |  |
|---|---|---|---|---|---|---|-----------------------|--|
| Specificity of the Ragweed Inhibiting Substance |   |   |   |   |   |   |                       |  |
| Test subject                                    | Mixture for sites *                       |   |   |   |   |   | Timothy units per cc. | Reaction to test with 1/40 cc. timothy 1,000 units per cc. |
|   | Serum combination in equal amounts        |   |   |   |   |   |                       |  |
| R. S.   | Timothy Serum A and ragweed Serum A (Sc.) |   |   |   |   |   | 25                    | +  |
|   | "   | " | " | " | " | " | 50                    | 0  |
|   | "   | " | " | " | " | " | 100                   | 0  |
|   | "   | " | " | " | " | " | 150                   | 0  |
|   | "   | " | " | " | " | " | Saline                | +++  |
|   | Timothy Serum A and ragweed Serum P (Sc.) |   |   |   |   |   | 25                    | +  |
|   | "   | " | " | " | " | " | 50                    | 0  |
|   | "   | " | " | " | " | " | 100                   | 0  |
|   | "   | " | " | " | " | " | 150                   | 0  |
|   | "   | " | " | " | " | " | 300                   | 0  |
|   | "   | " | " | " | " | " | 500                   | 0  |
|   | "   | " | " | " | " | " | Saline                | +++  |

+ = extent of skin reaction.  
0 = negative skin reaction.

\* 1/10 cc. of these mixtures of serum combinations and timothy extract or serum combinations and saline was placed in each site.

mixtures in the skin of non-sensitive test subjects. In 48 hours the sites were tested with timothy extract. Table V is a typical example of these experiments.

This experiment yielded no evidence of any inhibiting effect by the ragweed immune substance even against as closely related a reaction as that produced by timothy pollen and its specific serum on normal skin cells. Such experiments must be widely extended with many allergens and their specific serums to establish absolute specificity of

the inhibiting or immune substance. Our results thus far indicate specificity.

The final point which we have considered in working toward an explanation of the primary inhibition of the skin reaction (at the time the mixtures are injected) when ragweed was mixed with Serum P, deals with the question of a possible neutralization of the theoretical histamine-like substance. Two points already brought out indicate that this is not the case. The H-substance of Lewis (20), if responsible for these specific reactions, must be produced as a result of the action of allergen on the sensitized cell. There was no specific reaction when the mixtures of Serum P and ragweed were injected into normal skin because there was a reaction when the sites were tested after 48 hours. If there had been a reaction as with Serum A the sites when tested 48 hours later would have been negative.

Again if these specific reactions were due to H-substance, and if absence of reactions with allergen-Serum P mixtures were due to an antihistamine effect in Serum P, then it should follow that the inhibiting effect of Serum P would be non-specific; but we have already shown that the inhibition is specific for ragweed as against timothy allergen. A direct approach to the solution of this question has been made by testing the skin of normal persons and allergic patients with a solution of histamine in Serum A and in Serum P. The comparative reactions were so similar that there was no evidence of any antihistamine substance in Serum P.

#### DISCUSSION

In an interpretation of the facts brought out by these comparative studies of Serums A and Serums P the crucial point is to explain the inhibition of the reaction with the Serum P mixtures in normal skin at the time the sites are made. The cell is there, the allergen is there, and we have proven by titration that practically as much and sometimes even more of the skin sensitizing antibody is there. Then why no reaction? We have shown that there is no binding, no inactivation or lysis of the allergen. We have also explained that results cannot be interpreted as neutralization of H-like substance. What is evident is that injection of allergen-Serum P mixtures into skin produces no specific reaction at the time the sites are made, but the skin sensitizing antibody is found sensitizing the skin cells at the site when tested 48

hours later. It seems then that we must assume a block by some sort of specific inhibiting antibody. The block does not occur between the cell and the sensitizing antibody since the cells are later found sensitive. It must occur between the allergen and the sensitizing antibody, but since the test of the site is positive at the end of 2 days we must also assume that both inhibiting substance and allergen are shortly removed from the site. Also it is evident that the block is not absolute but may be overcome by the use of a sufficiently strong allergen in the mixture. In other words, the capacity to inhibit may be roughly measured.

Assuming, as we feel we have shown, the presence of a specific substance which blocks the antigen from the sensitized cell, the question may properly be raised whether this is the explanation of the clinical immunity afforded by specific treatment in this type of allergy. While it may not be the complete answer it satisfies many of the requirements. It permits one to understand the existing sensitization, shown by positive skin tests, while there is symptomatic freedom and it explains the occurrence of general reactions during treatment from dosage that will override the block. A more exacting test will be the determination that the amount of symptomatic freedom is proportional to the amount of inhibiting substance found. Studies to determine this point are being made.

#### SUMMARY

Using ragweed hay fever as the representative of a certain type of allergy we have made studies to determine if possible the mechanism of the protection afforded by specific injections thus far established only by clinical observation.

1. Blood transfusions and serum injections from clinically immune, treated patients stopped the clinical reaction in untreated patients, thus indicating a transferable immunity.

2. The amount of skin sensitizing antibody in the serum was found to be practically unchanged by specific injections.

3. Injection of allergen-antibody mixtures into normal skin showed an immediate (1 hour) reaction when sites were made if serum of untreated cases (Serum A) was used but none or slight reaction if serum of treated cases (Serum P) was used.

4. When sites made with allergen-antibody mixtures were tested

# IMMUNITY AND SENSITIZATION IN ALLERGY

after 48 hours, reactions were absent with Serum A mixtures if enough allergen had been used, but were positive with mixtures of Serum P even though a much stronger allergen was contained in the mixture.

5. The primary inhibition of reactions with mixtures including Serum P was not due to antihistamine effect nor to binding of skin sensitizing antibody nor to binding or lysis of allergen.

6. The inhibiting antibody appears to be specific.

7. These serological studies supported by transfusion experiments have been interpreted by us as showing the development under treatment of a peculiar blocking or inhibiting type of immune antibody that prevented the action of allergen on the sensitizing antibody and hence showed in the type of human allergy under consideration (hay fever) the coexistence of sensitization and immunity.

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## EXPLANATION OF PLATE 31

The figures illustrate Experiment 26 and the results recorded in Table II.

FIG. 1. 1 hour reactions when sites made, Apr. 12, 1935.

FIG. 2. Reactions when sites tested, Apr. 15, 1935.

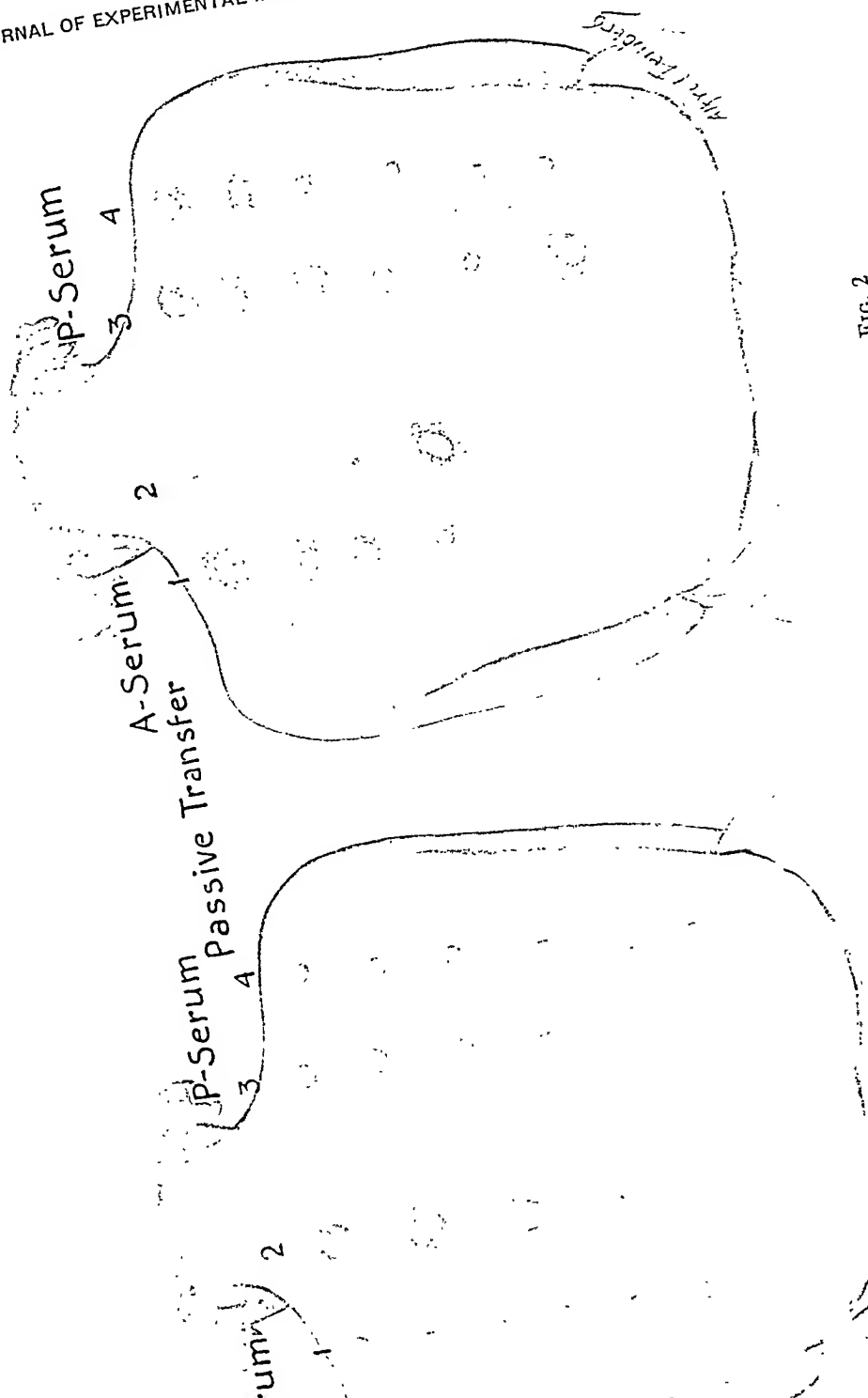


FIG. 2





# CELLULAR REACTIONS TO WAX-LIKE MATERIALS FROM ACID-FAST BACTERIA

THE UNSAPONIFIABLE FRACTION FROM THE TUBERCLE BACILLUS,  
STRAIN H-37

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PLATES 32 AND 33

(Received for publication, July 9, 1935)

The Mycobacteria are discriminated as a group by their capacity for synthesizing large amounts and varied types of lipoids in the form of fatty acids, phosphatides, and wax-like substances. Since no sterols have been found in them (1), the materials which have the properties of waxes are not waxes in the chemical sense. Rather they have been found to be composed of higher solid alcohols (2), and hydroxy acids of high molecular weight combined with polysaccharides, or, in the case of the corresponding substances from the *Bacillus leprae*, glycerides (3, 4). Like waxes, however, these materials are not only completely insoluble in water, but they cannot even be wet with water, properties which offer difficulties to the study of their effects on cells. Nevertheless, it can be shown that they are powerful stimulants for the new growth of cells. These wax-like materials have the property of acid-fastness and contribute it to the bacilli. For convenience we shall speak of these compounds as waxes.

As is well known, the separation of mixtures of lipoids is only to be made by the appropriate use of solvents. In general, Anderson has found that with the first use of each solvent, some of all the different types of lipoids come out. When it is recalled that almost all of the early biological tests of tuberculo-lipoids were made upon original alcohol-soluble, or alcohol-ether-soluble, or chloroform-soluble extracts, it is easy to understand why the effects so induced were complex and brought out no single cell reactions. Dr. Anderson has separated these lipoids from each other and given us three types of material.

The first, the so called acetone-soluble material, is a mixture of many fatty acids and is so irritating that it stimulates every type of connective tissue cell (5). The other two preparations, on the other hand, the phosphatides and the waxes, give practically a single cell reaction. Both of these materials stimulate monocytes, but their effect on the monocyte is different. With the phosphatide, monocytes become epithelioid cells and form tubercles in all their essential characteristics; while on the other hand, the waxes bring about a fusion of monocytes into foreign body giant cells. This paper is concerned primarily with the reactions to the unsaponifiable material or wax which will be considered in comparison with those to the phosphatide.

### *Materials and Methods<sup>1</sup>*

*The Unsaponifiable Wax from Human Tubercle Bacilli, H-37.*—This wax was obtained by Anderson (2) from the bacterial residue remaining after the extraction with alcohol-ether. The residue was treated with chloroform and the extract, after evaporation of the chloroform, gave a large amount of crude wax. This was then dissolved in ether, to which either acetone or methyl alcohol was added. A precipitate formed which was then separated into a saponifiable and an unsaponifiable portion by boiling in alcoholic potassium hydroxide. It is the unsaponifiable the chloroform extract. Some of this unsaponifiable material was also obtained from the original ether-alcohol extract both in the process of purification of the phosphatide and from the acetone-soluble material. After purification it is a white, amorphous powder, soluble in ether or chloroform, insoluble in water, and extremely stable. It is an hydroxy acid with the formula  $C_{94}H_{188}O_4$  (1).

In all of the earlier work in this laboratory with the waxes, including the crude wax as well as the more purified, the materials were introduced in solution in mineral oil (6). Since mineral oil itself, the most inert oil yet found, causes considerable reaction by inducing a new growth of fibroblasts, by inducing adhesions, and by being phagocytized by clasmatocytes (macrophages), we have now devised other methods of introducing the waxes. The intraperitoneal route has been used for the most part, but the material has also been introduced subcutaneously, intradermally, and intravenously.

The wax has been injected intraperitoneally in three ways: first, as a dry

<sup>1</sup> All of the materials we have used in these experiments have been obtained from the analyses of Dr. R. J. Anderson and his coworkers, Sterling Chemistry Laboratory, Yale University, and the work is a part of a plan for cooperative research sponsored by the Research Committee of the National Tuberculosis Association, of which Dr. William Charles White is Chairman.

powder through a cannula and either pushed in by trocar or blown in with air; second, as a dry powder through an incision under anesthesia; and third, in colloidal suspension. The first method proved the least instructive, because the wax-like materials frequently became packed into a single bolus within the needle. The second method is the most satisfactory for all of the different materials. It is possible, if aseptic precautions are used, to open the peritoneal cavity several times and dust the powdered waxes onto the omentum. The crude waxes, which were in large lumps, could be ground into fine powder if they were first chilled with dry ice until brittle. The wax often became charged electrically when scraped from the watch glass into the peritoneal incision. This, however, did not alter the cellular reactions.

The colloidal suspensions proved instructive on account of the opportunity they afforded to introduce these inert materials in fine particles. They were made by one of us as follows: A given weight of the unsaponifiable wax from H-37 was dissolved in chloroform and then an equal amount of hot alcohol was added. The material remained in solution. When an equal amount of distilled water was added drop by drop, the wax came out in a precipitate of particles so fine as to make a milky suspension. The flask was then placed in a water bath and kept at 100°C. until all of the chloroform and alcohol had been driven off. The water was then concentrated until 1 cc. contained 5 mg. of the wax. This suspension proved to be stable with no aggregation of the precipitate into clumps on standing.

## RESULTS

### *Reactions to Unsaponifiable Wax from H-37 Given Intraperitoneally.*—

The unsaponifiable material from tubercle bacilli, H-37, has been given intraperitoneally to seventeen rabbits in amounts shown in Table I. All of these animals showed also the reaction to the subcutaneous injection, since small amounts always lodged there in introducing the wax. In the table the animals are arranged according to the time which elapsed from the first injection to the date of killing the animal.

In the peritoneum the reaction to the wax is under the mesothelium of the parietal peritoneum, in the serosal coat of the cecum, in the capsule of liver and spleen, and in the omentum. The descriptions in the table are mainly of the omentum as representative of the entire reaction, because of the advantages in studying this structure. It can be studied as a film, while the cells are living, showing the supravital reaction to neutral red and Janus green. Then the same preparation can be fixed and stained in some manner, such as with the Ziehl-

## WAX-LIKE MATERIALS FROM ACID-FAST BACTERIA

TABLE 1  
*Protocols of Rabbits Which Received Unsaponifiable Wax from Tubercle Bacillus, H-37, Intraperitoneally*

| Protocols of Rabbits Which Received Unsaponified -LIKE MATERIALS FROM ACID-FAST B. |                              |              |  |                    |        |       |  |
|--|------------------------------|--------------|--|--------------------|--------|-------|--|
| Animal No.   | No. and amount of injections | Time         | Method of preparing material                 | Peritoneal exudate |        |       | Tissues  |
|  |                              |              |  | Percentage of      |        |       |  |
|  |                              |              |  | PMN                | Lymph. | Mono. |  |
| R 3351*  | 1<br>24 mg.                  | days<br>1 K† | Colloidal suspension                         | 41.5               | 2.6    | 55.8  | Acid-fast material on milk spots of omentum and none in interspaces. 10% of monocytes in peritoneal exudate contain acid-fast material show an increase in monocytes Milk spots of omentum have not started to fuse into surrounding wax, but they have not started to fuse into surrounding body giant cells. Infiltration with eosinophilic foreign body giant cells. Wax became highly charged electrically and lodged in fat leucocytes around the kidney, where foreign body giant cells developed. No infiltration with eosinophilic leucocytes around the omentum. Foreign body giant cells in omentum Died of pneumonia. |
| R 2760   | 1<br>20 mg.                  | 4<br>K       | Dry powder through cannula                   | 17.1               | 8.8    | 74.1  |  |
| R 2793   | 1<br>20 mg.                  | 4<br>K       | Dry powder through incision under anesthesia | 2.55               | 34.7   | 62.75 |  |
| R 2840   | 2<br>20 mg.                  | 4<br>2       | "  | —                  | —      | —     |  |
| R 2884   | 1<br>20 mg.                  | 6<br>K       | "  | —                  | —      | —     |  |
| R 3301   | 2<br>20 mg.                  | 4<br>3<br>K  | Colloidal suspension                         | 3.3                | 19.8   | 76.85 | Wax in small particles on milk spots, surrounded by newly formed monocytes which are starting to fuse into foreign body giant cells. Coccidiosis present. Reaction Milk spots show dense masses of monocytes around the milk some have fused into foreign body giant cells. Small foci of to acid-fast stain brilliant; it is in part free on the cells. Infiltration with eosinophilic leucocytes. Small foci of monocytes in septa between air sacs in lungs   |

[illegible]

PMN = polym  
\* These are ser  
† K = Killed.  
† D = Died.

## WAX-LIKE MATERIALS FROM ACID-FAST BACTERIA

TABLE I—*Concluded*

WAX-LIKE MATERIALS FROM ACID-FAST

Tissues

TABLE

| Animal No. | No. and amount of injections | Time   | Method of preparing material                 | Peritoneal exudate |        |       | Tissues   |
|------------|------------------------------|--------|--|--------------------|--------|-------|---|
|            |                              |        |  | Percentage of      |        |       |   |
|            |                              |        |  | PMN                | Lymph. | Mono. |   |
| R 2806     | 2<br>20 mg.                  | 4 days | Dry powder through incision under anesthesia | 0                  | 46.5   | 53.0  | Milk spots increased in size with monocytes and foreign body giant cells. 8 to 10 giant cells in each milk spot. Moderate infiltration with eosinophilic leucocytes. Sections show tubercles of giant cells<br>Milk spots of omentum not increased in size but show foreign body giant cells which, as well as the monocytes, show small acid-fast granules surrounded by a pink zone in Ziehl-Neelsen stain. Most of the reaction was in the retrosternal lymph nodes, where the sinuses were filled with small giant cells having a uniformly vacuolated cytoplasm<br>Milk spots of omentum massive with foreign body giant cells. Only the giant cells show any acid-fast material and this consists of tiny granules stained red and surrounded by a pink zone in Ziehl-Neelsen |
|            |                              | 23 K   |  | 0                  | 5.1    | 94.9  |   |
| R 3323     | 1<br>20 mg.                  | 30 K   | Colloidal suspension                         |                    |        |       | Milk spots of omentum dense with foreign body giant cells, some of which have irregular acid-fast particles that stain faintly with tubercles of foreign body giant cells; tissues infiltrated with lymphocytes and with some eosinophilic leucocytes. Cytoplasm of the giant cells is vacuolated. There is some calcification but nothing that simulates caseation   |
|            |                              |        |  |                    |        |       |   |
| R 3325     | 1<br>20 mg.                  | 32 K   | "  |                    |        |       | Milk spots of omentum dense with foreign body giant cells, some of which have irregular acid-fast particles that stain faintly with tubercles of foreign body giant cells; tissues infiltrated with lymphocytes and with some eosinophilic leucocytes. Cytoplasm of the giant cells is vacuolated. There is some calcification but nothing that simulates caseation   |
|            |                              |        | 0.5  | 17.35              | 82.15  |       |   |
| R 3326     | 1<br>20 mg.                  | 46 K   | "  |                    |        |       | Milk spots of omentum dense with foreign body giant cells, some of which have irregular acid-fast particles that stain faintly with tubercles of foreign body giant cells; tissues infiltrated with lymphocytes and with some eosinophilic leucocytes. Cytoplasm of the giant cells is vacuolated. There is some calcification but nothing that simulates caseation   |
|            |                              |        | 1.03   | 2.06               | 96.90  |       |   |
| R 2839     | 1<br>20 mg.                  | 55 K   | Dry powder through incision under anesthesia |                    |        |       | Milk spots of omentum dense with foreign body giant cells, some of which have irregular acid-fast particles that stain faintly with tubercles of foreign body giant cells; tissues infiltrated with lymphocytes and with some eosinophilic leucocytes. Cytoplasm of the giant cells is vacuolated. There is some calcification but nothing that simulates caseation   |
|            |                              |        |  |                    |        |       |   |

|        |             |               |        |   |       |       |   |
|--------|-------------|---------------|--------|---|-------|-------|---|
| R 2845 | 2<br>20 mg. | 4<br>133<br>K | "<br>" | 0 | 8.0   | 91.0  | Milk spots of omentum massive with foreign body giant cells. Similar masses in the body wall. All of the tissues infiltrated with some eosinophilic leucocytes; some calcification. Nodules of monocytes and eosinophilic leucocytes in the lung  |
| R 2844 | 2<br>20 mg. | 6<br>222<br>K | "<br>" | 0 | 56.65 | 43.35 | Milk spots of omentum massive with tubercles of foreign body giant cells. Some masses of giant cells raised that the wax had become much more finely divided in the pedicles. Supravital reaction to neutral red showed in the cells than in R 2845. Some plasma cells and an occasional eosinophilic leucocyte |



Neelsen technique. Other preparations of the omentum can be fixed for sections. Besides occurring in these locations, some of the wax, occasionally much of it, floods through the lymphatics of the diaphragm into the retrosternal lymph nodes. In most instances, small particles also reach the lung and are there represented by small foci of monocytes usually infiltrated with eosinophilic leucocytes.

The immediate reaction to the material injected in the form of the colloidal suspension is instructive. The material is identified by its property of acid-fastness.

The colloidal suspension itself shows only a diffuse, pink reaction to the fuchsin of the Ziehl-Neelsen technique, for the wax has to be in aggregates of a given size before the typical red color of the reaction can be obtained. Aggregates of the material are formed when the colloidal suspension is introduced intraperitoneally and the acid-fast reaction is shown in Fig. 1, from the omentum of Rabbit R 3351 which was killed 24 hours after one injection of 24 mg. This film was fixed in the vapor of formalin and stained with the Ziehl-Neelsen method. The wax, stained red with the fuchsin, appears black in the photograph and is almost limited to the milk spots.

It was clear on studying the fresh films of the omentum of this animal, R 3351, that the wax was merely adherent to the cells of the milk spots and had not been phagocytized by them. The cells of the peritoneal exudate were stained for acid-fast material and only 10 per cent of the monocytes contained any of this material and they only in small amounts.

In the fifth column of Table I is shown the differential counts of the cells of the peritoneal exudate expressed in percentages of the three strains of cells which are significant. In some of the counts there was an occasional basophilic leucocyte, never more than 2 per cent; but in none was there even a single eosinophilic leucocyte; in some counts there were a few clasmotocytes with phagocytized leucocytes and occasionally desquamated serosal cells. These cell types may be considered as accidental and hence have been omitted in the table. Only the first two animals listed in Table I showed any rise in neutrophilic leucocytes in the peritoneal exudate. In the case of Rabbit R 3351, the blood cells were counted hourly after the injection and by 4 hours the neutrophilic leucocytes had risen from 4,860 to 11,023 per c.mm.

Thus there is an immediate draining of neutrophilic leucocytes into the peritoneal cavity but it is not lasting unless infection supervenes. Rabbit R 3351 did not show any infiltration of the tissues with eosinophiles, which subsequently becomes a constant finding.

By 4 days, all of the animals receiving the wax have shown a multiplication of monocytes around the particles of the wax, but we have not found them fusing into giant cells until the 6th day. The omentum of Rabbit R 3301, which was studied 7 days after the first injection, showed the milk spots increased in size and dense with monocytes; most of the milk spots had giant cells, some as many as thirty. All the giant cells were small because the material was introduced in a fine suspension, the size of the foreign body giant cell being proportional to the size of the mass it has engulfed. The omentum stained for acid-fast material gave a brilliant result. The acid-fast material was almost all on the milk spots in masses from about 5 to 20 $\mu$  in diameter. The largest masses were surrounded by monocytes not yet fused into giant cells. All of the giant cells were filled with the acid-fast material. In sections of the omentum the giant cells showed a vacuolated cytoplasm indicating the zones from which the wax had been dissolved out in the processes of embedding. This explains why it is impossible to obtain positive acid-fast stains in sections of this material, because the free waxes are so easily removed by lipoidal solvents. The milk spots of the omentum were infiltrated with considerable numbers of eosinophilic leucocytes, but practically no neutrophilic leucocytes. In spite of this, there were no eosinophiles found free in the peritoneal exudate.

A later stage of the process is shown in Figs. 2, 3, and 4, representing 17, 27, and 32 days after the first injection. All the figures show giant cells of the omentum, photographed while the cells were living. All of the black color in the photographs represents the neutral red in the vacuoles that contain the wax. An acid-fast stain shows the wax itself; the supravital neutral red shows the fluid that the cell has secreted around the wax. It is clear that with the longer time, Fig. 3 at 27 days, much more wax has been phagocytized than was present at 17 days, Fig. 2. The reaction shown in Fig. 3 is of small tubercles of giant cells.

Three of the rabbits, R 3323, 3325, and 3326, which received the material in colloidal suspension gave suggestion of the fate of the wax within the cells.

These animals were killed 30, 32, and 46 days respectively after a single injection of the material in colloidal suspension. When the fresh omenta of these three animals were stained in the Ziehl-Neelsen technique, it was noted that instead of the massive red staining of the giant cells seen in Rabbit R 3301, killed 7 days after the injection, the reaction had now been modified. The giant cells of the first two animals, 30 and 32 days after the injection, all had diffuse, pink-stained zones in the center of which were tiny, bright red granules, while in the cells of the third animal, killed at 46 days, only the diffuse pink reaction remained. The type of giant cell seen in the omentum of Rabbit R 3325 is shown in Fig. 4. This cell is stained in neutral red; it is the type that showed the tiny, red granules surrounded by a diffuse, pink zone when stained for acid-fastness. It will be noted that the vacuoles of the cell, dark in the photograph, are irregular in size, but many of them, especially in the lower part of the cell, are small. In the cells of Rabbit R 3326, which had lost all of the material staining typically acid-fast, the cytoplasm of the living cells showed that the wax had become much more finely divided. Indeed, the cytoplasm reminded one of masses of platelets showing the same clumping of tiny granules.

These observations indicate that the wax becomes broken up in the cytoplasm of the giant cells until it is too finely divided to give an acid-fast stain, just as occurs when the wax is put into colloidal suspension.

In the case of Rabbit R 3323, most of the wax had flooded into the retrosternal lymph nodes, where the sinuses were distended with foreign body giant cells and the follicles had been much encroached upon by them. In these giant cells, the vacuoles were all small, giving evidence that the cells had been breaking the wax into small particles. Around these giant cells were many eosinophilic leucocytes. The omentum had so little of the reaction that the milk spots, though they showed a few giant cells, were not increased in size by them. In Rabbit R 3325, on the other hand, the milk spots were massive with giant cells.

The last three animals in the group, Rabbits R 2839, R 2845, and R 2844, killed 55, 137, and 228 days after the first injection of the wax, all had massive tubercles of foreign body giant cells in the omentum. Some of the milk spots were so thick that they were markedly raised from the surface of the omentum and some were even on pedicles. Within the giant cells there had been a progressive breaking up of the wax into smaller particles.

The type of giant cells is shown in Figs. 5 and 6 from the omentum of Rabbit R 2844. Fig. 5 is a foreign body giant cell of enormous size; the photograph is at low magnification, namely, 400 diameters. The cell has over a hundred nuclei. Smaller giant cells with from three to six nuclei are seen in the neighborhood. The cytoplasm is rather uniformly mottled, showing places from which the wax has been dissolved out in embedding. This is not as marked in sections as in the living cell on account of the shrinkage suffered during embedding.

Fig. 6 is a part of a tubercle of giant cells; again there is a great range in the size of these cells. One of the giant cells shows a central cavity from which the wax has been dissolved out. In the fresh preparations a few of the giant cells still showed unchanged wax in the center and this is evidently one of them. It will be noted in Figs. 5 and 6 that there are no signs of necrosis in any of the cells. All of the nuclei appear normal. This is characteristic of the sections throughout. In the sections of this animal there are many zones, such as the one marked with an arrow in Fig. 6, in which there is the suggestion that this type of giant cell may split into its component monocytes when the wax has become sufficiently finely divided. The history of the view that certain giant cells in tuberculous tissue do not regress by necrosis but rather break up into smaller cells was reviewed by Hektoen (7) in 1898, who brought evidence in support of this view from the study of a case of tuberculous peritonitis.

*Reactions to Unsaponifiable Wax from H-37 Given Intravenously.*—In Table II are given the protocols of eleven rabbits which received the unsaponifiable wax intravenously, five of which were subsequently inoculated with bovine tubercle bacilli.

The first five animals received the wax in doses of 3 mg. suspended in mineral oil and all showed foci of oil droplets in the septa between air sacs. In these zones there was an increase in monocytes which had phagocytized the oil.

Rabbit R 3350 received six intravenous injections of the wax in colloidal suspension in water and was killed 32 days after the first injection. In this manner all of the complicating factors from the menstruum were removed and the reaction to the wax came out. Throughout the liver there were many giant cells in the sinuses, two to four in every oil immersion field. There was no reaction around them; no eosinophilic leucocytes were found. Some of these giant cells had only two or three nuclei; they were simple in type, with the nuclei in the periphery and the cytoplasm vacuolated in the center. Others had as many as thirty nuclei; these were complex, having two or three cytoplasmic centers surrounded by nuclei in rings or with clumps of them along the border. In almost all of these giant cells the cytoplasm was vacuolated. In the spleen there were many small giant cells, both in the sinuses and in the follicles; many of the follicles had small, tubercle-like bodies of monocytes. Also many of the follicles had small patches of early amyloid degeneration; occasionally a follicle was almost replaced by this type of degeneration. There were no more eosinophilic leucocytes in the spleen

TABLE II  
*Protocols of Rabbits Receiving Unaponifiable Wax from Tubercle Bacillus, H-37, Intravenously*

| Animal No. | No. and amount of injections | Time   | Method of preparing material   | Inoculation with bovine tubercle bacilli | Tissues  |
|------------|------------------------------|--|--------------------------------|--|--|
|            |                              |  |                                |  |  |
| R 2001     | 2<br>3 mg.                   | 1 wk. apart. K<br>24 hrs. after second injection | Dissolved in 1 cc. mineral oil | —  | <p>Many foci of oil droplets, monocytes, and neutrophilic leucocytes in septa between air sacs in lungs. Tracheal lymph nodes were filled with leucocytes and the spleen had many clasmatocytes engorged with occasional highly vacuolated mononuclear cells in septa between air sacs. Foci of monocytes and leucocytes, with occasional highly vacuolated mononuclear cells in septa between leucocytes in lungs. Some of these foci large enough to obliterate a few air sacs. Spleen shows much destruction of leucocytes</p> <p>Many foci of monocytes and leucocytes with some highly vacuolated mononuclear cells and a few foreign body giant cells in lungs. Some large enough to obliterate 20 air sacs. Spleen shows a few small, tubercle-like masses of monocytes</p> <p>Larger foci of highly vacuolated mononuclear cells in lungs. These foci are vascularized and contain a few free leucocytes. Liver shows extreme involvement with cocci cytes. Liver shows foci of necrosis</p> <p>diosis and spleen has foci of necrosis. A few Foci of cells in lungs, 2 to 3 mm. in diameter, consisting mainly of highly vacuolated mononuclear types. No dead leucocytes and many eosinophilic leucocytes. No destruction of leucocytes in the spleen but some foci of monocytes</p> |
|            |                              | 1 wk. apart. K<br>24 hrs. after third injection  | "                              | —  |  |
| R 2002     | 3<br>3 mg.                   | 1 wk. apart. K<br>24 hrs. after third injection  | "                              | —  |  |
|            |                              | 1 wk. apart. K<br>24 hrs. after fourth injection | "                              | —  |  |
| R 2003     | 4<br>3 mg.                   | 1 wk. apart. K<br>7 days after fourth injection  | "                              | —  |  |
|            |                              | 1 wk. apart. D<br>7 days after fourth injection  | "                              | —  |  |
| R 2004     | 4<br>3 mg.                   | 1 wk. apart. K<br>2 days after sixth injection   | "                              | —  |  |
| R 2006     | 6<br>3 mg.                   |  |                                |  |  |

|  |   | Daily except last interval was 2 days. K 25 days after sixth injection | Colloidal suspension 12 mg. of wax per 1 cc. water | —   |   |
|--|---|--|--|---|---|
| R 3350   | 6<br>12 mg.<br>24 "<br>12 "<br>10 "<br>8 "<br>8 " |  |  |   | Many small, foreign body giant cells in sinuses throughout the liver. Many foreign body giant cells in spleen both in sinuses and in follicles. Few giant cells in septa between air sacs in lungs and in glomeruli of kidney. No infiltration with eosinophiles  |
| R 2006<br>R 2007<br>R 2008<br>R 2009<br>R 2010 | — "<br>74 6<br>3 mg.                              | 1 wk. apart  | Dissolved in 1 cc. mineral oil                     | 0.1 mg. Strain B-1, intravenously, 2 days after sixth injection | Survived an average of 176 days, with a range of 57 to 306 days, as compared with a survival of 162 days, range of 30 to 287 days for 15 controls (R 2073-R 2087). There were no differences in the type of disease in the rabbits which had received the wax in oil, but they all had foci of highly vacuolated mononuclear cells in lungs; these foci did not become invaded with the tuberculous process |

*Tubercle Bacillus, H-37, Intravenously*

TABLE II

*Protocols of Rabbits Receiving Unsaponifiable Wax from*

Tissues\*

| Animal No. | No. and amount of injections | Time  | Method of preparing material      |   | Inoculation with bovine tubercle bacilli | Tissues*   |
|------------|------------------------------|---|-----------------------------------|---|--|--|
|            |                              |   |                                   |   |  |  |
| R 2001     | 2<br>3 mg.                   | 1 wk. apart.<br>24 hrs. after<br>second injection | Dissolved in 1 cc.<br>mineral oil | — | —  | Many foci of oil droplets, monocytes, and neutrophilic leucocytes in septa between air sacs in lungs. Tracheal lymph nodes were filled with leucocytes and the spleen had many clasmatocytes engorged with occasional highly vacuolated mononuclear cells in septa between air sacs. Foci of monocytes and leucocytes, with occasional highly vacuolated mononuclear cells in septa between air sacs. Some of these foci large enough to obliterate in lungs. Spleen shows much destruction of leucocytes. Many foci of monocytes and leucocytes with some highly vacuolated mononuclear cells and a few foreign body giant cells in lungs. Some large enough to obliterate 20 air sacs. Spleen shows a few small, tubercle-like masses of monocytes |
|            |                              | 1 wk. apart.<br>24 hrs. after<br>third injection  |                                   |   |  |  |
| R 2002     | 3<br>3 mg.                   | 1 wk. apart.<br>24 hrs. after<br>third injection  | "                                 | " | —  | Larger foci of highly vacuolated mononuclear cells in lungs. These foci are vascularized and contain a few free leucocytes. Liver shows extreme involvement with cocci-<br>diosis and spleen has foci of necrosis consisting of cells in lungs, 2 to 3 mm. in diameter, consisting of highly vacuolated mononuclear types. No mainly of highly vacuolated mononuclear leucocytes. No dead leucocytes and many eosinophilic leucocytes. destruction of leucocytes in the spleen but some foci of monocytes  |
|            |                              | 1 wk. apart.<br>24 hrs. after<br>fourth injection |                                   |   |  |  |
| R 2003     | 4<br>3 mg.                   | 1 wk. apart.<br>24 hrs. after<br>fourth injection | "                                 | " | —  | Foci of cells in lungs, 2 to 3 mm. in diameter, consisting of highly vacuolated mononuclear types. No mainly of highly vacuolated mononuclear leucocytes. No dead leucocytes and many eosinophilic leucocytes. destruction of leucocytes in the spleen but some foci of monocytes  |
|            |                              | 1 wk. apart.<br>7 days after<br>fourth injection  |                                   |   |  |  |
| R 2004     | 4<br>3 mg.                   | 1 wk. apart.<br>7 days after<br>fourth injection  | "                                 | " | —  | Foci of cells in lungs, 2 to 3 mm. in diameter, consisting of highly vacuolated mononuclear types. No mainly of highly vacuolated mononuclear leucocytes. No dead leucocytes and many eosinophilic leucocytes. destruction of leucocytes in the spleen but some foci of monocytes  |
|            |                              | 1 wk. apart.<br>2 days after<br>sixth injection   |                                   |   |  |  |
| R 2006     | 6<br>3 mg.                   | 1 wk. apart.<br>2 days after<br>sixth injection   | "                                 | " | —  | Foci of cells in lungs, 2 to 3 mm. in diameter, consisting of highly vacuolated mononuclear types. No mainly of highly vacuolated mononuclear leucocytes. No dead leucocytes and many eosinophilic leucocytes. destruction of leucocytes in the spleen but some foci of monocytes  |
|            |                              | 1 wk. apart.<br>2 days after<br>sixth injection   |                                   |   |  |  |

Many small, foreign body giant cells in sinuses throughout the liver. Many foreign body giant cells in spleen both in sinuses and in follicles. Few giant cells in septa between air sacs in lungs and in glomeruli of kidney. No infiltration with eosinophiles

Survived an average of 176 days, with a range of 57 to 306 days, as compared with a survival of 162 days, range of 30 to 287 days for 15 controls (R 2073-R 2087). There were no differences in the type of disease in the rabbits which had received the wax in oil, but they all had foci of highly vacuolated mononuclear cells in lungs; these foci did not become invaded with the tuberculous process

|        | Daily except last interval was 2 days. K 25 days after sixth injection | Colloidal suspension 12 mg. of wax per 1 cc. water | —   |  |
|--------|--|--|---|--|
| R 3350 | 6<br>12 mg.<br>2½ "<br>12 "<br>10 "<br>8 "<br>8 "<br>— "<br>74         |  |   |  |
| R 2006 | 6  | Dissolved in 1 cc. mineral oil                     | 0.1 mg. Strain B-1, intravenously, 2 days after sixth injection |  |
| R 2007 | 3 mg.  |  |   |  |
| R 2008 |  |  |   |  |
| R 2009 |  |  |   |  |
| R 2010 |  |  |   |  |



## WAX-LIKE MATERIALS FROM ACID-FAST BACTERIA

than normally. An occasional small giant cell was found in the lung and also in the glomeruli of the kidney. The blood cells of this animal were studied, and from the time of the fourth injection until the animal was killed, the eosinophilic leucocytes were above 500 per c.mm., and twice they were above 1,000. The average number of eosinophiles in the normal rabbit is 110 per c.mm.

The last five animals on Chart 2 received six injections each of 3 mg. of the wax in mineral oil at intervals of 1 week and, 2 days after the last injection, were inoculated intravenously with 0.1 mg. of bovine tubercle bacilli, Strain B-1. At the same time, fifteen rabbits were inoculated with the same dose from the same suspension for controls. There were no significant differences in longevity between the injected animals and their controls, nor in the range of survival. All of the animals which had received the wax had small foci of vacuolated cells in the septa between air sacs, but these foci did not become invaded with the tubercle bacilli, resulting in setting up tuberculous masses, but even then they were not these foci became surrounded by epithelioid cells. Thus there was no sign that these abnormal foci of cells in the lungs had any effect whatever on the tuberculous infection.

## DISCUSSION

The most interesting biological property of these solid alcohols and hydroxy acids which make the unsaponifiable material of *Mycobacteria* and contribute the property of acid-fastness to them is that, though they are stable and seemingly inert substances chemically, they are remarkable stimulants for the new growth of cells. Their essential property is that they stimulate the formation of monocytes wherever they lodge in the tissues. These monocytes then fuse around the wax and become foreign body giant cells.

It is clear that whenever lipoids are introduced parenterally, they are dealt with by the phagocytic mononuclear cells, that is, by monocytes. For this study the omentum gives the most valuable data; after intraperitoneal injection, it is easy to see that lipoids lodge on the milk spots of the omentum and not in the interspaces between them. This has been illustrated in Fig. 1 by means of the acid-fast property of the tuberculo-wax. When the lipid is introduced in the form of a uniform colloidal suspension, we must assume that the fluid passes both through milk spots and interspaces, but only the part of the wax which floods through the milk spots becomes fixed (Fig. 1). This must be either because of the greater density of the primitive cells and monocytes which make up the milk spots, or on account of

the characteristics of their surface films. Tiny foci of young, connective tissue cells, so readily identified as milk spots in the omentum, exist throughout the connective tissues, and their property of fixing lipoids to their surface and then phagocytizing them may well be the source of the monocytes in the local formation of tubercles.

We have previously studied the effect of another lipid especially characteristic of tubercle bacilli, namely, a phosphatide (6, 8-10), on the cells of the connective tissues. The phosphatide, in contrast to the waxes, is readily dispersed in water, making stable suspensions which are suitable for parenteral injection. In water these phosphatides form myelin-like figures (10) which can be readily identified within cells. It is thus possible to show that these phosphatides are phagocytized by monocytes and that they are acted upon within the cell in a specific manner. The material is at first irregular in size, making a cell that appears highly vacuolated in fixed material, since the processes of treating tissues for sectioning result in a complete solution of the lipid. However, it is soon broken up into small and then smaller particles within the cytoplasm. This process seems to go on uniformly so that the particles are all about the same size at any stage, except immediately after phagocytosis. When they have become finely divided, the cell is the typical epithelioid type, indistinguishable from the form seen in the disease tuberculosis. The time necessary for the formation of epithelioid cells ranges from 4 days to 2 weeks. Some of the monocytes which have phagocytized the phosphatide become multinuclear, making Langhans' giant cells.

The only constituent of the phosphatide capable of bringing about this reaction is a fatty acid, discovered by Anderson and named by him phthioic acid. This acid is optically active and has the formula of  $C_{26}H_{52}O_2$ . It has not been found in nature before and is highly characteristic of tubercle bacilli. It occurs in all of the three major lipid fractions as first obtained through solvents, namely, in the alcohol-ether-soluble phosphatide, in the acetone-soluble mixture of fatty acids, and in the chloroform-soluble material (1, 11-13). When this fatty acid is given either in the form of salts (5), or with its burning properties cut down by suspension in nujol, it forms typical epithelioid cells, singly and in tubercles (8-10).

The fate of the epithelioid cell is an essential point in judging its

meaning; after the introduction of the lipoid either as phosphatide or as fatty acid, it is broken up into fine particles, after which no further change can be detected in the cell, except that refractive bodies may appear in the periphery. The end-result, though it may be long delayed, is the death of the cell. Thus the monocyte can readily phagocytize this material and disperse it to a certain state, but it seems not physiologically adapted to a disintegration of this fatty acid into its simpler molecular groups. Epithelioid cells then regress, either through the death of the individual cells, or *en masse*, in which case the phenomenon is called caseation (10).

The giant cells produced in response to the waxes must be compared with the epithelioid types. The unsaponifiable material from H-37, which is a higher hydroxy acid with the formula  $C_{94}H_{188}O_4$ , can be identified within the cells by the property of acid-fastness. Only the finest particles of this material, as when introduced in colloidal suspension, are phagocytized by the individual monocyte; rather the masses of the wax become surrounded by many monocytes which fuse into a giant cell which is at first a hollow sphere. The large inner surface of this fused cytoplasm is then able to engulf the material.

When the wax is sufficiently finely divided, as in the colloidal suspension, it gives only a diffuse pink reaction with the Ziehl-Neelsen technique, but when the small particles again become aggregated, to follow the action of the giant cells upon the wax. Whether the material has been introduced in colloidal suspension or in powder form, it appears within a period of 2 to 3 weeks as massive aggregates which are acid-fast. The material remains for a long time within the giant cell in the form of irregular masses, with no immediate tendency toward the formation of particles of equal size, so characteristic of the epithelioid type. But in the period of months, typical acid-fastness gradually grows less, until only a diffuse, pink reaction like that of the colloidal suspension remains. This indicates that the cell is able to disperse this material into finer particles, such as are in the colloidal suspensions. In sections the giant cells which were originally coarsely vacuolated become much more finely vacuolated, as is shown in Figs. 5 and 6. If such a giant cell is small and has peripheral nuclei, as is shown in Fig. 5, it is indistinguishable from the typical Langhans' type.

Thus the method of dealing with the wax within the giant cells is different from that of dealing with the phosphatide. It is a slower process and there is nothing that might be compared to a process of emulsification as with the phosphatide, but there is a gradual disintegration of the material until it is too finely divided to give an acid-fast stain.

More important still is the difference in the fate of the cell which has taken in the wax. We have not detected any signs of damage to the cells; they do not disappear through caseation; there is no sign of cell death in the tissues. It is probable that the giant cells eventually separate into single monocytes, though our experiments have not been carried far enough to prove this point.

The first reaction to the introduction of the wax into the peritoneal cavity is the calling of neutrophilic leucocytes into the peritoneal cavity, but this follows the introduction of any foreign material whatever. It is a reaction which does not persist, and in about 2 weeks' time the neutrophilic leucocytes have disappeared from the tissues and eosinophilic leucocytes appear. This reaction is so marked that it can be detected by an increase in eosinophilic myelocytes in the bone marrow and, if the blood cells are followed, by an increase in their number in the blood stream. They do not seem to wander into the peritoneal exudate but rather remain around the giant cells. Their presence is so constant around these lesions that they suggest that the giant cells break some material from the wax which is chemotactic to eosinophiles. Some of the foci of giant cells, especially those that form quite large tubercles, also become surrounded or infiltrated with lymphocytes. It is not a constant reaction, tubercles of giant cells in any one section varying as to whether there are few, many, or no lymphocytes around them.

The study of the effect of the unsaponifiable material injected intravenously on the course of a subsequent infection with tubercle bacilli indicates that the waxes have no effect on resistance. These materials are elaborated by the *Mycobacteria* and give them the property of acid-fastness by which they are classified, but their function is probably in connection with the life and the survival of the bacillus and they induce no reactions in the host that are associated with resistance.

## CONCLUSIONS

1. The unsaponifiable fractions of the Mycobacteria, though insoluble in water and extremely stable chemical compounds, are nevertheless remarkable stimulants of cells.
2. They give rise to new monocytes which surround these waxes and then fuse into giant cells which engulf them.
3. The property of acid-fastness of the waxes makes it possible to identify them within the giant cells which have phagocytized them.
4. Within the foreign body giant cells the waxes are slowly disintegrated. They appear not to damage the cells which engulf them, and hence one may infer that they take no part in caseation.
5. They have no effect on the resistance of the host.

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## EXPLANATION OF PLATES

## PLATE 32

FIG. 1. Film of omentum of Rabbit R 3351, which had received one intraperitoneal injection of 24 mg. of the unsaponifiable material from human tubercle bacilli, Strain H-37, suspended in 2 cc. of water. Killed 24 hours after the injection. Film stained with the Ziehl-Neelsen technique to bring out acid-fast material which shows as black masses against the milk spots.  $\times 10$ .

FIG. 2. Foreign body giant cell in the center of a milk spot of the omentum, photographed while the cells were living, of Rabbit R 2814, which had received two intraperitoneal injections of the unsaponifiable material from the human

tubercle bacillus, H-37, and was killed 14 days after the second injection. The material was introduced as a dry powder through an incision under anesthesia. The film is stained with neutral red and was photographed while the cells were still living. The material stained black represents the fluid around the particles of wax, which had been phagocytized; there is only a small amount of this reaction within the giant cell; there are well stained monocytes in the border. The refractive bodies seen around the giant cell are in the slightly stimulated serosal cells covering the milk spot.  $\times 1040$ .

FIG. 3. Small tubercle of foreign body giant cells in a film of omentum of Rabbit R 2806, which had received two intraperitoneal injections of the unsaponifiable material from the human tubercle bacillus, H-37, and was killed 23 days after the second injection. The material was introduced as a dry powder through an incision under anesthesia. The film is stained with neutral red and was photographed while the cells were still living. The material stained black represents the fluid around the particles of wax, which had been phagocytized, and shows a marked increase from the stage of Fig. 2. The wax is in relatively large masses.  $\times 693$ .

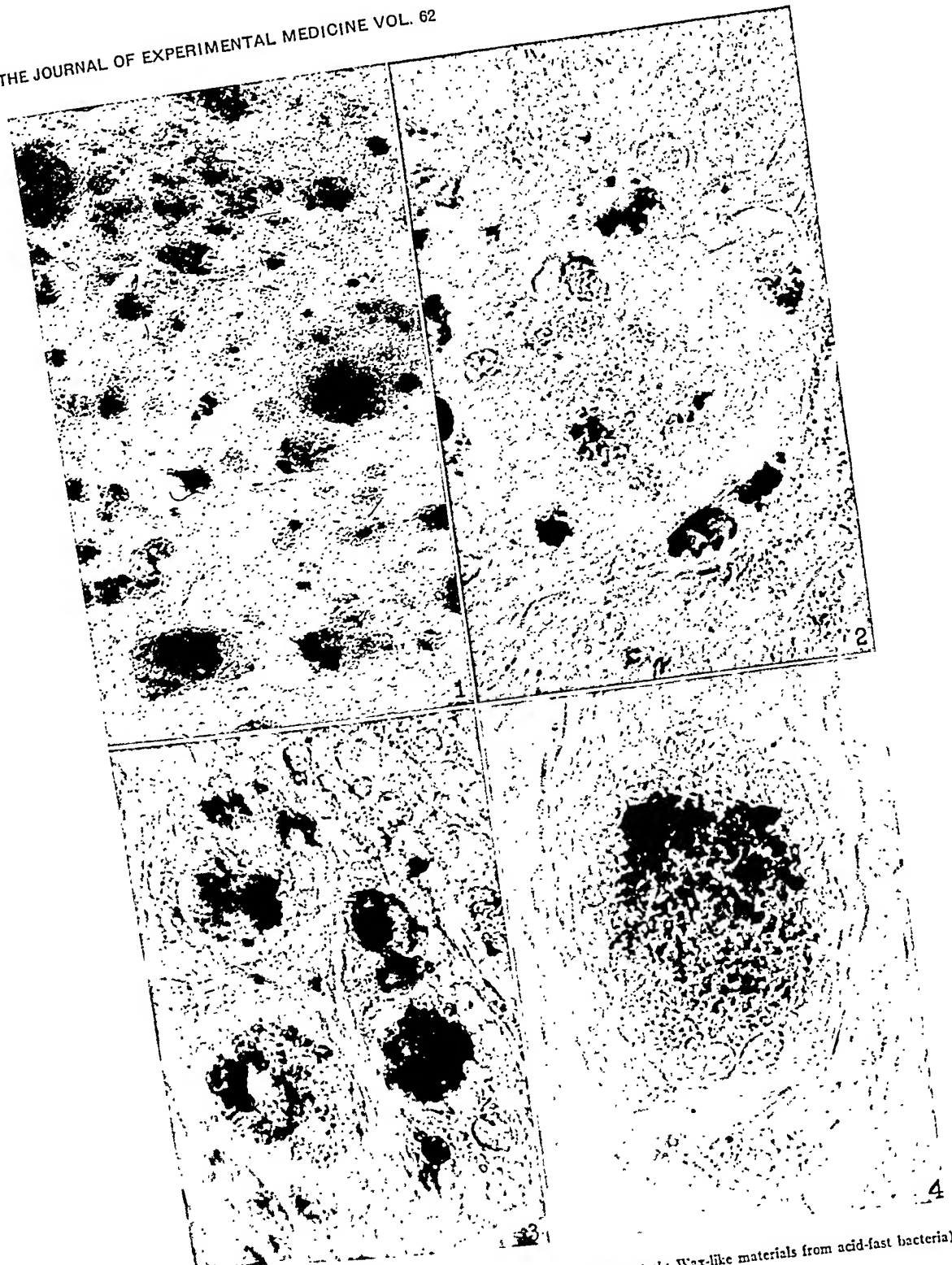
FIG. 4. Foreign body giant cell in a film of omentum of Rabbit R 3325, which had received one intraperitoneal injection of the unsaponifiable material from human tubercle bacillus, H-37, and had been killed 32 days later. The material was introduced in colloidal suspension in 3 cc. of water. The film was stained with neutral red and photographed while the cells were still living. The material which shows as black (neutral red) represents the fluid secreted by the cell around the particles of the wax and indicates that some of the wax is still in large aggregates while some is in fine particles.  $\times 866$ .

#### PLATE 33

FIG. 5. Large and small foreign body giant cells in a section of the omentum of Rabbit R 2844, which had received two injections of the unsaponifiable material from the human tubercle bacillus, H-37, and had been killed 222 days after the second injection. Stained with hematoxylin and eosin.  $\times 400$ .

FIG. 6. Tubercles of foreign body giant cells in a section of the omentum of the same animal as in Fig. 5. The arrow points to a zone in which a giant cell may be breaking up into its component monocytes. Stained with hematoxylin and eosin.  $\times 400$ .



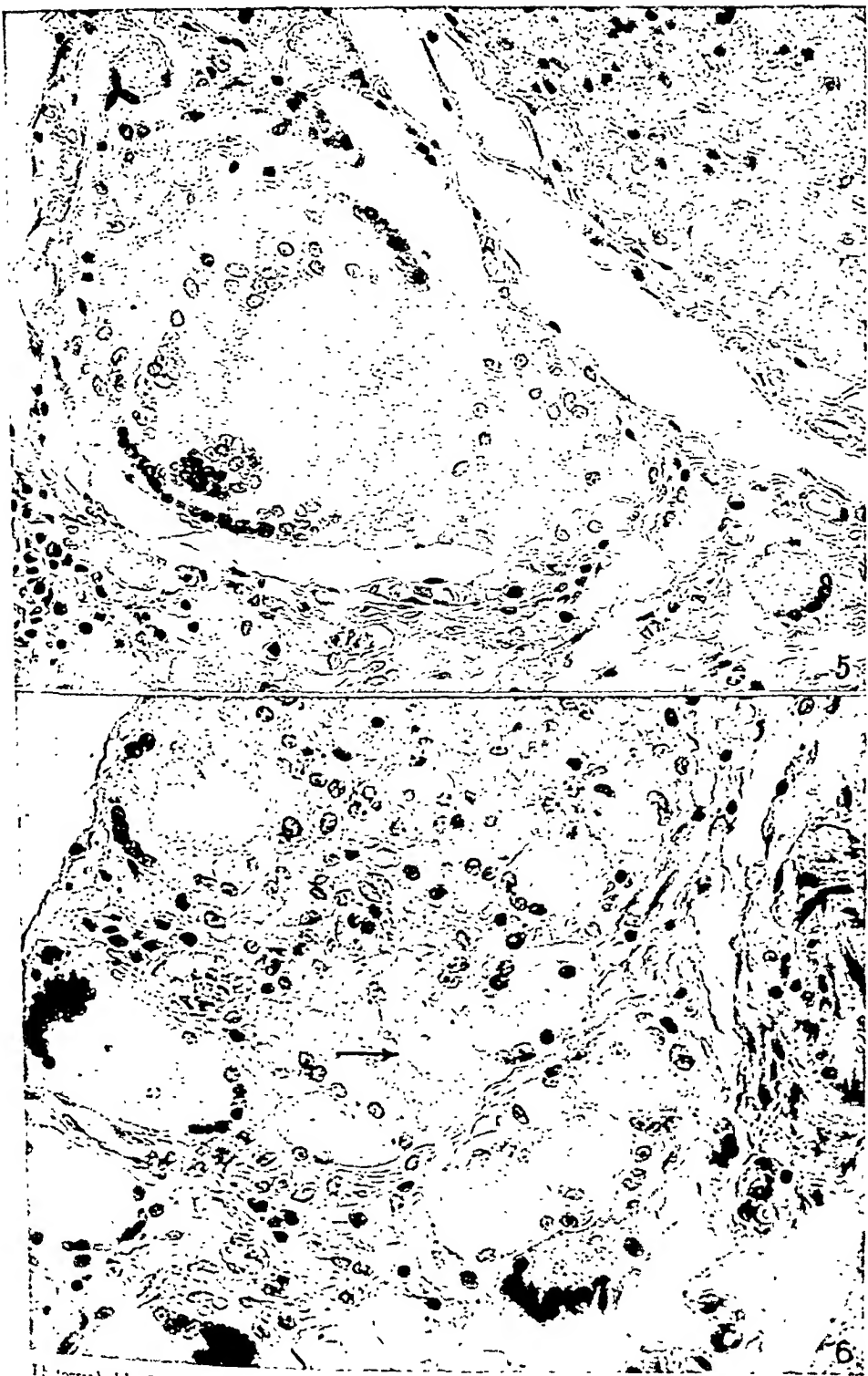


11 \* prepared by Louis Schmidt

(Sabin *et al.*: Wax-like materials from acid-fast bacteria)







Photographed by Louis Schmidt

(Sabin *et al* : Wax-like materials from acid-fast bacteria)



## CELLULAR REACTIONS TO WAXES FROM MYCOBACTERIUM LEPRAE

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PLATE 34

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The plan for chemical analysis of the *Mycobacteria* sponsored by the National Tuberculosis Association included an acid-fast strain isolated from a case of leprosy, as well as selected strains of human, bovine, and avian tubercle bacilli, and this study deals with the waxes extracted from *Mycobacterium leprae*.<sup>1</sup> The chemical analyses have not yet been completely reported (1, 2). The materials used have been in different stages of purification and have therefore provided the opportunity to compare the complex cellular reactions to mixtures of substances with the simple response to highly purified crystalline alcohols.

### *Materials and Methods*

The strain of organisms used for the analysis was obtained from a case of leprosy (Apa case) in Honolulu, about 1909. It has been kept at the Hygienic Laboratory, Washington, D. C., as Strain 370; on Feb. 4, 1926, a subculture was given to the Mulford Biological Laboratories, Glenolden, Pennsylvania. It was there grown on Long's synthetic medium in quantities adequate for chemical analysis.

Five preparations of wax fractions from the *Bacillus leprae* have been studied.

1. *A Crude Chloroform-Soluble Wax*.—This was obtained in the same manner as the corresponding fraction from tubercle bacilli (3). This fraction has not yet been analyzed completely, but Dr. Anderson reports that it is almost free from phosphorus and nitrogen and hence is not a phosphatide (1). It is a red, wax-like substance, looking like beeswax except for the color.

2. *A Crude Wax Obtained from the Purification of the Lepra Phosphatide*.—This material could be removed from the crude phosphatide because it is more

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<sup>1</sup> We are indebted for this material to Dr. R. J. Anderson and his associates at Yale University, who extracted the waxes from *Mycobacterium leprae*.

soluble in a mixture of ether and acetone than the phosphatide, and it came out on cooling the acetone solution (1). Concerning this product, Dr. Anderson has written us, "This product consists mainly of a solid glyceride which I call 'Leprosin.' It contains a number of fatty acids and the alcohols  $C_{20}H_{42}O$  and  $C_{18}H_{38}O$ , identical with the alcohols isolated from the wax of the timothy grass bacillus." This material is a slightly pink, amorphous powder; that is, it carries some of the pigment so characteristic of the *B. leprae*.

3. *Leprosin*.—This was obtained from the purification of the preceding material. Though a glyceride instead of an alcohol, it corresponds in a general way in its physical properties to the unsaponifiable material from tubercle bacilli. It is a snow white powder, with a melting point of  $54^{\circ}C$ . It is optically active.

$$[\alpha]_D^{25} \text{ in chloroform } + 4.2^{\circ}.$$

4. *Leprosinic Acid*.—This was obtained from the analysis of the leprosin and in physical properties is like it. It has a melting point of  $61-62^{\circ}C$ . and is dextrorotatory:

$$[\alpha]_D^{25} \text{ in chloroform } + 4.7^{\circ}.$$

All four of these materials from the *B. leprae* are acid-fast.

5. *Alcohol II from the Unsaponifiable Matter of the B. leprae*.—This is a pure white material consisting of feather-like crystals. It has a melting point of  $84^{\circ}C$ . and a formula of  $C_{25}H_{44}O_2$ . It is not acid-fast.

Besides this alcohol from the *B. leprae* we have also received from Dr. Anderson a similar material as follows:

*Alcohol Isolated from the Wax of the Timothy Grass Bacillus*.—This is a pure white material made up of fine, needle-like crystals. Its melting point is  $63^{\circ}C$ . It is dextrorotatory:

$$[\alpha]_D^{25} \text{ in ether } + 6.93^{\circ}.$$

Its formula is  $C_{20}H_{42}O$ . It is not acid-fast.

None of these preparations has been given in nujol, the method used in our first study of wax fractions from tubercle bacilli (4). Rather it has been found better to introduce these materials in the form of a dry powder through an incision under ether anesthesia. The crude waxes can be ground into a fine powder if they are first chilled with dry ice until brittle. The more purified substances, the leprosin, the leprosinic acid, and the alcohols, do not need more than simple grinding to separate the clumps of crystals.

The method of preparing these waxes from the *B. leprae* in colloidal suspension, which was developed by one of us with the unsaponifiable material from the tubercle bacillus (5), was tried with the lepra fractions but with less success. It consisted of dissolving the wax in chloroform and adding an equal amount of hot alcohol. To this solution the same amount of distilled water was added drop by

drop, which procedure threw down the wax in the form of a precipitate. The suspension obtained from the crude chloroform-soluble wax was not as fine as that from the unsaponifiable material from the tubercle bacillus but rather consisted of particles about  $7\mu$  in diameter which settled out into quite large aggregates. This material, therefore, had to be freshly prepared for each injection.

When the crude wax obtained from the purification of the lepra phosphatide was prepared for colloidal suspension, the precipitate was of more or less regular pentagonal particles, highly refractive and suggesting a crystalline form. There was no agglutination of these particles.

In attempting to prepare similar suspensions of leprosin and leprosinic acid, the addition of distilled water resulted in the separation of minute droplets of an oily nature which formed an unstable emulsion. This emulsion had a tendency to curdle and adhere to the surface of the flask. This was in marked contrast to the stability of the suspension prepared from the unsaponifiable material from H-37.

## RESULTS

*Reactions to the Crude Chloroform-Soluble Wax from B. leprae.*—The crude chloroform-soluble wax from the *B. leprae* was given intraperitoneally to six rabbits, as shown in Table I.

Rabbits R 4268 and R 4269 both received the material after it had been powdered with dry ice. The material did not give a simple reaction; where the wax had lodged, the area became infiltrated with leucocytes making an abscess. Around these abscesses were monocytes, fibroblasts, and clasmotocytes, filled with leucocytes. In the omentum the reaction was not uniform; there were abscesses, bands of fibroblasts, and foci of monocytes, some showing partial fusion into giant cells. There was a marked dilatation of the vessels. The retrosternal lymph nodes had their sinuses filled with monocytes and in some places they were present also in the follicles.

The same tendency toward the formation of abscesses followed the introduction of the material in colloidal suspension.

This preparation showed a marked tendency to a clumping of the particles, with the result that the dose varied; Rabbit R 4189 received a small dose, R 4187 an average dose, and R 4188 a massive dose. The abscesses were smaller because the masses of the wax had been smaller. They were filled with clasmotocytes containing leucocytes. Around the abscesses were monocytes and giant cells, many of them vacuolated, indicating a phagocytosis of the wax. The edge of one of the abscesses with a narrow border of giant cells is shown in Fig. 1, from Rabbit R 4188. Bands of fibroblasts were extensive; in one animal of the group, R 4188, there was an extreme involvement of the retrosternal lymph nodes with

TABLE I  
*Protocols of Rabbits Which Received the Crude Chloroform-Soluble Wax from B. leprae*

WAXES FROM MYCOBACTERIUM TUBERCULOSIS

Tissues

Most of the wax in one bolus. Coccidiosis present

Many abscesses, 2 to 3 cm. in diameter in omentum, on cecum, in the peritoneal wall, and in incision. Abscesses surrounded by monocytes and fibroblasts, with clasmatocytes containing leucocytes in the border. Omentum almost too massive to study as film and very complex zones of fibroblasts; many small foci of monocytes showing partial fusion into giant cells. Marked center of which contain leucocytes. Retrosternal nodes have vascular dilatation. Many abscesses 2 to 3 cm. in diameter in omentum surrounded by monocytes, fibroblasts, and clasmatocytes containing leucocytes. Many small foreign body giant cells with vacuolated fusion of the plasm. Diaphragm shows tubercles of giant cells in which there is only partial fusion of the monocytes. Leucocytes infiltrating of the Bands of fibroblasts; marked dilatation of type vessels. Extensive reactions of the same type in retrosternal nodes replacing part of the follicles

Protocols of Rabbits Which Received the Crude

| Animal No. | No. and amount of injections | Time   | Method of preparing material  | Peritoneal exudate |        |       | Tissues   |
|------------|------------------------------|--------|---|--------------------|--------|-------|---|
|            |                              |        |   | Percentage of      |        | Mono. |   |
|            |                              |        |   | PMN                | Lymph. |       |   |
| R 2795     | 1<br>20 mg.                  | 4<br>K | Dry powder through cannula  | —                  | —      | —     | Most of the wax in one bolus. Coccidiosis present |
| R 4268     | 1<br>50 mg.                  | 6<br>K | Material powdered with dry ice and introduced through incision under anesthesia | 15.0               | 14.0   | 70.80 |   |
| R 4269     | 1<br>50 mg.                  | 6<br>K | "   | 63.68              | 0      | 36.31 |   |

|        |             |  |                      |      |       |       |  |
|--------|-------------|--|----------------------|------|-------|-------|--|
| R-4187 | 3<br>20 mg. | 4<br>K 5 days after<br>third injection | Colloidal suspension | 1.78 | 22.19 | 76.02 | No symptoms. No rise in temperature. Small abscesses in body wall surrounded by fibroblasts and clasmatocytes containing leucocytes. Omentum has no large abscesses but many small tubercles of foreign body giant cells and monocytes, some vacuolated. In the center of the tubercles were leucocytes. Dilatation of the vessels and marked bands of fibroblasts. Few giant cells in retrosternal lymph node. One small hemorrhage in one lung, infiltrated with monocytes   |
| R-4188 | 3<br>20 mg. | 4<br>K 5 days after<br>third injection | "                    | 8.88 | 24.36 | 66.75 | No symptoms. No rise in temperature. Abscesses 4 to 10 mm. in diameter on surface of cecum, in capsule of liver and spleen, on diaphragm, in body wall, and in the omentum. Abscesses contain clasmatocytes filled with leucocytes and are surrounded by a narrow band of monocytes. Omentum has many small tubercles of foreign body giant cells and monocytes, some of them vacuolated. Dilatation of vessels and bands of fibroblasts. Lungs had several small, translucent nodules which were made up of monocytes. Retrosternal nodes show extreme involvement with monocytes and giant cells both in the sinuses and in the follicles. In the fresh tissue, typical epithelioid cells were seen, confirmed in sections |
| R-4189 | 3<br>20 mg. | 4<br>K 5 days after<br>third injection | "                    | 0.25 | 42.45 | 57.28 | Very slight reaction; a few abscesses in the body wall and a few foreign body giant cells in the omentum   |



TABLE II

Protocols of Rabbits Which Received the Wax Obtained in the Purification of the Phosphate of *B. leprae*

| WAXES FROM MYCOBACTERIUM                    |                              |                |   |  |        |       |
|---|------------------------------|----------------|---|--|--------|-------|
| Tissues                                     |                              |                |   |  |        |       |
| Protocols of Rabbits Which Received the Wax |                              |                |   |  |        |       |
| Animal No.                                  | No. and amount of injections | Time           | Method of preparing material  | Peritoneal exudate   |        |       |
|   |                              |                |   | Percentage of  |        |       |
|   |                              |                |   | PMN  | Lymph. | Mono. |
| R 2715                                      | 1<br>20 mg.                  | days<br>3<br>K | Dry powder through cannula blown in with air                                      | 1.02   | 5.10   | 93.87 |
| R 2800                                      | 1<br>20 mg.                  | 4<br>K         | Dry powder through incision under anesthesia                                      | 1.13   | 1.13   | 97.72 |
| R 2798                                      | 2<br>20 mg.                  | 4<br>D         | Dry powder through cannula. Died after second injection which punctured the liver | —  | —      | —     |
|   |                              |                |   | Omentum showed increased size of milk spots with particles of wax on them. Increase in monocytes; occasional fusion into giant cells, some having as many as 50 nuclei. Few epithelioid cells. Infiltration with neutrophilic leucocytes and a few myelocytes. No increase in fibroblasts. Retrosternal node has many monocytes in the sinuses. Omentum showed increased size of milk spots with particles of wax on them. Around the particles of omentum showed increased size of milk spots with particles of wax on them. No signs of phagocytosis; that is, no vacuoles staining with eosinophilic and eosinophilic neutral red. Few neutrophilic and eosinophilic leucocytes. No increase in fibroblasts. Wax in single giant cells in omentum. Blood in foreign body giant cells in omentum. Blood in retrosternal lymph nodes 5 min. after puncture of the liver |        |       |

|        |             |  |  |                  |   |
|--------|-------------|--|--|------------------|---|
| R 2763 | 2<br>20 mg. | 4<br>K 3½ hrs. after<br>second in-<br>jection        | Dry powder through<br>cannula blown in<br>with air | Cells all dead   | 3 hrs. after the second injection the animal had a convulsion, was extremely sensitive on being touched, became cyanotic, pulse was about 250 and respiration was slow. Temperature was 111.5° and the animal was killed. Small abscesses in mesentery, diaphragm, and omentum. Omentum showed increased density of the milk spots with monocytes and giant cells, dilatation of the vessels, neutrophilic leucocytes, both free and in clasmatocytes, and a few epithelioid cells. Marked damage of the lymphocytes in the nodes, probably due to the temperature. Congestion of the lungs and liver. Hemorrhage in the bone marrow  |
| R 4184 | 3<br>20 mg. | 4<br>4<br>K 6 days after<br>the third in-<br>jection | Colloidal suspension<br>in 4 cc. water             | 5.59 45.29 49.10 | No symptoms; no rise in temperature. Small abscesses on peritoneal wall near places of injections, on the large intestine, on the diaphragm, and in the omentum. Milk spots of omentum increased in size and number. Abscesses of omentum contain many clasmatocytes filled with leucocytes and have a wide border of giant cells. Many giant cells in tubercles and diffusely scattered. Marked vascularization, bands of fibroblasts, and increase in fibrous tissue. Acid-fast stain negative. Retrosternal nodes show extreme numbers of monocytes in the sinuses and the follicles are almost replaced by them. Small, translucent nodules in lungs made up of monocytes |

WAXES FROM MYCOBACTERIUM LEPRÆ

TABLE II—Concluded

WAXES FROM MYCOBACTERIUM

Tissues

TABLE II

| Animal No. | No. and amount of injections | Time                               | Method of preparing material        | Peritoneal exudate | Tissues |       |  |  |
|------------|------------------------------|------------------------------------|-------------------------------------|--------------------|---------|-------|--|--|
|            |                              |                                    |                                     | Percentage of      |         |       |  |  |
|            |                              |                                    |                                     | PMN                | Lymph.  | Mono. |  |  |
| R 4185     | 3<br>20 mg.                  | days                               | Colloidal suspension in 4 cc. water | 27.62              | 18.31   | 54.05 | No symptoms. No rise in temperature. Lesions like those of R 4184, except less reaction in the fibers in the omentum and less reaction of monocytes in retrosternal nodes. Nodules of monocytes in the lungs after the first injection. After the second, rapid respiration and temperature fell 2.1° and then rose 3.3°. After the third the temperature fell 2.5° and then rose 4.8°. One of the injections had lodged in the sheath of the external oblique muscle, where there was a nodule of giant cells, and monocytes, many of them vacuolated; very few neutrophilic leucocytes but many eosinophilic. Omentum showed increased density of the milk spots due to monocytes and giant cells, whose cytoplasm was filled with vacuoles. Some wax still to be seen on the milk spots. Some typical epithelioid cells. Moderate increase in fibroblasts |  |
|            |                              | 4                                  |                                     |                    |         |       |  |  |
|            |                              | 4                                  | Dry powder through cannula          | 5.20               | 13.54   | 81.25 |  |  |
|            |                              | K 6 days after the third injection |                                     |                    |         |       |  |  |
| R 2771     | 3<br>20 mg.                  | 4                                  | K 5 days after the third injection  |                    |         |       |  |  |
|            |                              | 4                                  |                                     |                    |         |       |  |  |

monocytes and giant cells, as shown in Fig. 3 which is a section through the sinuses. The follicles of this node were also extensively replaced by similar cells. Besides these reactions, typical epithelioid cells were found in the omenta and the retrosternal lymph nodes of these animals, both with the supravital technique and in sections.

*Reactions to Wax from the Purification of the Phosphatide of B. leprae.*—The wax obtained from the purification of the lepra phosphatide was given to seven rabbits, as shown in Table II. This was the only material given which was followed by any symptoms and they proved to be inconstant.

The early reaction was shown by two rabbits, R 2715 and R 2800, one of which received the material through a cannula and the other through an incision. In neither animal were there symptoms after the injection. The wax had lodged on the milk spots of the omentum, and around the particles of the wax there had been a multiplication of monocytes; in places these monocytes had started to fuse into giant cells. There were occasional epithelioid cells but there was no increase in fibroblasts.

Rabbit R 2763 showed extreme disturbance after the second injection, 3 hours later it had a convulsion, the temperature rose to  $111.5^{\circ}$ , and the animal was therefore killed. A second rabbit, R 2771, had a rise in temperature of  $3.3^{\circ}$  after a third injection but survived. We did not determine the cause of the fever in Rabbit R 2763. The material injected showed no bacteria. The brain and cord were normal; there were small abscesses in the omentum, in the mesentery, and on the diaphragm, but not as many as in the rabbits of the preceding series. There was the same reaction of monocytes and giant cells around the wax which had lodged on the milk spots of the omentum.

Rabbit R 2771 showed similar cellular reactions, complex in type; there were foreign body giant cells around the particles of wax, a few neutrophilic leucocytes, and many eosinophilic leucocytes. There were epithelioid cells and an increase in fibroblasts.

The material was then given to two rabbits, R 4184 and R 4185, in three doses in colloidal suspension. None of these injections caused symptoms and there was no rise in temperature. In making the suspension, the material had been heated. There were small abscesses wherever the wax had lodged. The abscesses had wide borders of monocytes and giant cells, as is shown in Fig. 2. The width of the border of giant cells can be seen to be greater than in Fig. 1, which was taken from an animal which had received the crude chloroform-soluble wax. Indeed, almost the entire section shown in Fig. 2 is of monocytes except the abscess on the lower border. Besides this, the material from Rabbit R 4184 showed many large masses of giant cells, some in tubercles and some diffusely scattered, so that when the total reaction was compared with that in the animals

WAXES FROM MYCOBACTERIUM LEPRAE

TABLE III  
Protocols of Rabbits Receiving *Leprosin*

| Protocols of Rabbits Receiving Wax |                              |                                      |  |                    |        |       | Tissues   |
|------------------------------------|------------------------------|--------------------------------------|--|--------------------|--------|-------|---|
| Animal No.                         | No. and amount of injections | Time                                 | Method of preparing material                 | Peritoneal exudate |        |       |   |
|                                    |                              |                                      |  | Percentage of      |        | Mono. |   |
|                                    |                              |                                      |  | PMN                | Lymph. |       |   |
| R 2801                             | 2<br>20 mg.                  | 4<br>K 4 days after second injection | Dry powder through incision under anesthesia | 6.5                | 5.0    | 88.0  | No symptoms after either injection. Omentum shows marked increase in size of milk spots with foreign body giant cells enclosing the wax. Eosinophilic leucocytes in the retrosternal lymph nodes is an ex-culture. No epithelioid cells. Eosinophilic leucocytes in the predominant reaction of foreign body In omentum of tubercles of hollow centers and  |
| R 4264                             | 1<br>50 mg.                  | 7<br>K                               | Colloidal suspension                         | 5.0                | 13.50  | 80.75 | tensive cytoplasm; others have a vacuolated cyto-giant cells. Some have a |

| R 2812 | 2<br>20 mg. | 4<br>K 14 days<br>after the sec-<br>ond injec-<br>tion | Dry powder through<br>incision under anes-<br>thesia | — | —    | —     | No symptoms. Omentum shows foreign body<br>giant cells singly and in tubercles. The giant<br>cells have enclosed the wax and a few leucocytes.<br>Very little vacuolization of the cytoplasm of the<br>giant cells. Increase in fibroblasts and in bands<br>of fibers. A few small abscesses, but almost no<br>neutrophilic leucocytes elsewhere. No eosino-<br>philic leucocytes |
|--------|-------------|--|--|---|------|-------|---|
| R 2807 | 2<br>20 mg. | 4<br>K 24 days<br>after the sec-<br>ond injec-<br>tion | " " "  | 0 | 9.64 | 90.35 | Every milk spot of omentum has foreign body<br>giant cells, some extremely large. Their cyto-<br>plasm is markedly vacuolated. Tubercles of<br>giant cells seen in section. Many bands of<br>fibroblasts and many eosinophilic leucocytes in<br>the omentum. Many eosinophiles in the retro-<br>sternal nodes   |

that received the crude chloroform-soluble wax, it was clear that this preparation had a much greater proportion of the factor, that is the wax, that produces the foreign body giant cells. Besides the giant cells there was a marked dilatation of the blood vessels, seen also in Fig. 2, as well as signs of the new formation of fibroblasts and of fibrous tissue.

*Reactions to Leprosin.*—Five rabbits have received the leprosin and their protocols are given in Table III. The formation of abscesses, so marked a feature of the reaction to the two preceding materials, was much reduced, appearing only in Rabbit R 4264.

There were two constant reactions to the material, an extensive formation of foreign body giant cells and a marked development of bands of fibroblasts. These bands are shown in Fig. 4, from the omentum of Rabbit R 2801. The photograph was taken from a fresh film, stained with neutral red, and was made while the cells were living. It will be noted that the bands of fibroblasts are growing in a manner to simulate a tissue culture. The giant cells occurred singly and in tubercles and had eosinophilic leucocytes around them. No epithelioid cells were seen after the injection of the leprosin.

*Reactions to Leprosinic Acid.*—The cellular reactions to the intraperitoneal injections of leprosinic acid are shown in five rabbits, the protocols of which are given in Table IV. In every instance the reaction to this material has been of a single cell type; namely, there has been a multiplication of monocytes around the particles of the wax and their fusion into giant cells.

In the omentum it was clear that this material also lodged only on the milk spots which became tubercles of giant cells. The differential counts of the cells of the peritoneal exudates of these animals show how small a part of the reaction is made by neutrophilic leucocytes, for only in the first animal of the series were there any of these cells and in that instance, only 2.5 per cent. Instead the tissues were infiltrated with eosinophilic leucocytes.

*Reactions to Alcohols from the Waxes of the B. leprae and B. phlei.*—These two highly purified, crystalline alcohols,  $C_{26}H_{44}O_2$ , from the unsaponifiable wax material of the *B. leprae*, and  $C_{20}H_{42}O$ , from the *B. phlei*, or timothy grass bacillus, were each given intraperitoneally to two rabbits in doses of 15 mg. They were given in each instance as the dry crystals through an incision under ether anesthesia.

Rabbits R 4272 and R 4241 each received the alcohol from the *B. leprae* and were killed in 5 days. Rabbit R 4236 and R 4238 received the alcohol from the

TABLE IV

*Protocols of Rabbits Receiving Leprosinic Acid*

| Animal No. | No and amount of injections | Time    | Method of preparing material                 | Peritoneal exudate |        |       | Tissues   |
|------------|-----------------------------|---------|--|--------------------|--------|-------|---|
|            |                             |         |  | Percentage of      |        |       |   |
|            |                             |         |  | PMN                | Lymph. | Mono. |   |
| R 2769     | 1<br>20 mg.                 | 5<br>K  | Dry powder through cannula                   | 2.52               | 7.07   | 90.40 | <p>Omentum showed accentuated milk spots with wax enclosed in giant cells. Tubercles of foreign body giant cells on diaphragm infiltrated with eosinophilic leucocytes. Many eosinophiles in the retrosternal lymph nodes</p> <p>Omentum showed flecks of wax on the milk spots surrounded by foreign body giant cells. These giant cells were not as large as in R 4270 but there were as many as 30 to 40 on some milk spots. Foci of monocytes in septa of lungs and some giant cells in the retrosternal lymph nodes</p> <p>Omentum showed flecks of wax on the milk spots surrounded by foreign body giant cells. Some of them had only three or four nuclei; others were so large that they completely filled a low power field. Many monocytes; no leucocytes seen. No foci of monocytes found in the lungs</p> <p>Omentum showed many giant cells on the milk spots, some with 30 to 40 nuclei. There were 2.5% eosinophilic leucocytes in the peritoneal exudate and there was one giant cell about 100<math>\mu</math> in diameter. A focus of giant cells and monocytes near the point of injection was infiltrated with eosinophilic leucocytes. A few foci of monocytes in the lungs and extensive involvement of both sinuses and follicles of retrosternal lymph nodes with monocytes</p> <p>Less reaction than in R 4266 but of same type. Monocytes and foreign body giant cells containing wax on the milk spots of the omentum</p> |
| R 4271     | 1<br>30 mg.                 | 22<br>K | Dry powder through incision under anesthesia | 0                  | 14.81  | 85.18 |   |
| R 4270     | 1<br>30 mg.                 | 23<br>K | "  | 0                  | 13.40  | 86.59 |   |
| R 4266     | 1<br>30 mg.                 | 37<br>K | Imperfect colloidal suspension               | 0.51               | 15.97  | 83.50 |   |
| R 4267     | 1<br>30 mg.                 | 38<br>K | "  | 0                  | 14.79  | 85.20 |   |



## WAXES FROM MYCOBACTERIUM LEPRAE

timothy grass bacillus and were killed in 4 and 6 days respectively. The reaction was of giant cells around the crystals of alcohol; these crystals lodged on the milk spots of the omentum. The giant cells were proportional to the size of the crystals, whether they occurred singly or in a clump. There were more neutrophilic leucocytes in the peritoneal fluid than when the time interval had been longer, ranging from 5 to 11 per cent, except in the case of Rabbit R 4238, in which there was an adhesion of a part of the omentum to the peritoneal wall and to the liver, and the leucocytes were 39.08 per cent.

These experiments have not been completed; they involve a study of the reactions to these materials in the tuberculous rabbits, as well as in the normal ones.

## DISCUSSION

The materials from the lepra bacillus have given an interesting opportunity to follow the chemical separation of complex mixtures by cells instead of in the test tube. It is clear that all of these materials contain some substance which, like the solid alcohol  $C_{25}H_{44}O_2$ , gives the formation of the simple foreign body giant cell. The crude chloroform-soluble wax and the wax obtained from the purification of the lepra phosphatide contain, however, many more substances that give cellular reactions. With both of them the most striking phenomenon is the formation of large abscesses wherever the masses of the wax lodge. Thus there is some substance chemotactic to leucocytes in or on the wax which acts like the tuberculo-polysaccharide. That is, this material calls leucocytes from the blood stream and so damages them that they are readily engulfed by clasmatoocytes. The leucocytes are found in clasmatoocytes both in the local lesions and in the spleen. These abscesses are not at all like caseation for there is no basis of dead cells, but rather it is the wax itself which becomes infiltrated with leucocytes.

Besides this reaction, there is the formation of the foreign body giant cells with the fused monocytes enclosing both the wax and the leucocytes. In the case of the wax obtained from the analysis of the lepra phosphatide, this reaction is much greater in amount than with the crude chloroform-soluble wax, indicating that the wax-like material is in much greater proportion in this fraction. The tissues also become infiltrated with eosinophilic leucocytes and lymphocytes.

These materials also are irritants, causing a marked dilatation of the blood vessels and probably a new growth of vessels. They also contain substances that are remarkable stimulants for the formation of fibroblasts and of new fibers. Besides all of these properties, they contain some material that gives rise to typical epithelioid cells, exactly like the reaction to the tuberculo-phosphatide. Thus these two materials give reactions as complex as those aroused by the acetone-soluble material from the tubercle bacillus (6). The cellular reactions to them are similar to those aroused by the crude wax which can be separated from tubercle bacilli. These complex cellular reactions reflect the fact that some of all the different types of lipoids come out with the first use of lipoidal solvents on mixtures.

The leprosin is an entirely different type of material; it is a pure white powder in crystalline form which does not give the cellular reactions characteristic of complex mixtures of lipoids, such as have just been described. Rather its reactions are reduced to two simple properties: It causes a remarkable new growth of fibroblasts, as is shown in Fig. 4, making them grow as they do in tissue cultures. Then it produces the formation of foreign body giant cells around the particles of the wax, and subsequently gives an infiltration of the tissue with the eosinophilic leucocytes. With the leprosinic acid the material which causes the new growth of the fibroblasts has been split off from the molecule of the leprosin, and the resulting fraction causes only the formation of the foreign body giant cells. This property is seen in its purest state with the crystalline solid alcohol, in which each giant cell is proportional in size and shape to a single crystal or a clump of crystals which may happen to lodge on a milk spot.

#### CONCLUSIONS

1. The waxes from the *B. leprae*, like those from tubercle bacilli, are remarkable stimulants of cells.
2. The crude wax separated from the *B. leprae* is a mixture of lipoids and other materials, and gives reactions that include the types of cells characteristic of the response to the tuberculo-polysaccharide, phosphatide, and wax.
3. The wax obtained from the purification of the lepra phosphatide shows similar cellular reactions but with a greater proportion of foreign body giant cells.

4. Leprosin, though a glyceride, corresponds in its physical properties to the unsaponifiable material from the tubercle bacillus. It stimulates two strains of cells, fibroblasts and monocytes. The monocytes fuse into foreign body giant cells to engulf the wax.

5. The cellular reaction to the leprosinic acid and to the crystalline alcohols is of one type only, represented by the foreign body giant cell.

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## EXPLANATION OF PLATE 34

FIG. 1. Wall of an abscess in the omentum of Rabbit R 4188, which had received three intraperitoneal injections of 20 mg. each of the crude chloroform-soluble wax from the *B. leprae*. The wax was in the form of a colloidal suspension. The animal was killed 5 days after the third injection. There is a narrow band of monocytes and giant cells along the edge of the abscess. Masson stain to show the deep basophilia of the cytoplasm of the monocytes.  $\times 210$ .

FIG. 2. Wall of an abscess in the omentum of Rabbit R 4184, which had received three intraperitoneal injections of 20 mg. each of the wax obtained from the purification of the lepra phosphatide. The animal was killed 6 days after the third injection. There is a wide band of monocytes and giant cells along the edge of the abscess. Masson stain.  $\times 250$ .

FIG. 3. Sinuses of one of the retrosternal lymph nodes of the same animal as Fig. 1. It shows a pure and extensive reaction of monocytes and giant cells. Masson stain.  $\times 1,000$ .

FIG. 4. Film of the omentum of Rabbit R 2801, which had received two intraperitoneal injections of 20 mg. of leprosin in the form of a dry powder. The animal was killed 4 days after the second injection. The preparation was stained with neutral red and the photograph was taken while the cells were still living, and shows bands of fibroblasts.  $\times 150$ .



H. Longuetty-Louis-Schmidt

S. J. and C. Waxes (from *Myxobolus* sp.)



# THE EFFECTS OF NASALLY INSTILLED VIRUS OF POLIO-MYELITIS ON THE CEREBROSPINAL FLUID AND THE BLOOD OF MONKEYS

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While much has been written on the ultimate effects of neurotropic poliomyelitis virus dropped into the nose of *Macacus* monkeys and made to run over the olfactory nervous areas, little has been published on the immediate, remote influence of the presence of the virus on the nasal membranes.<sup>1</sup> The subject is one of real importance, if for no other reason than because of the commonly held belief that spontaneous mass immunization to poliomyelitis among children results from the chance presence in the upper respiratory tract of the widely disseminated virus, not only during the prevalence of epidemics, but even during interepidemic periods. That the nasal portal of entry of the virus is effective is shown by the frequency with which the paralytic disease arises among nasally instilled monkeys in the absence of

\* I desire to acknowledge with many thanks the efficient help given me by Mr. Peter Haselbauer in carrying out the experiments on which the series of articles on poliomyelitis now in course of publication is based.

<sup>1</sup> Jungeblut and Hazen (1) subjected four *rhesus* monkeys to "extensive" spraying of the nose and throat with a 10 per cent suspension of poliomyelitis virus during a period of 2½ months. The spraying was done twice a week, each animal receiving 20 sprays of about 1 cc. Three animals survived the test period, and were bled 1 month after the last treatment. The serum was tested for antiviral, and the monkeys were inoculated cerebrally with virus. All three monkeys developed paralytic poliomyelitis, and the sera exerted no detected neutralizing action. The doses of virus (1 cc. of a 10 per cent suspension), and the virus (0.5 cc. of a 1 per cent suspension) and serum (0.5 cc.) mixtures, indicate either a strain of low virulence, or the use of overwhelming quantities in the tests. However, the results seem unequivocal, inasmuch as Flexner (2), employing more measured amounts of virus, also reported that monkeys refractory to nasal instillation of virus were devoid of serum antiviral activity and exhibited average susceptibility to the intracerebral injection of potent virus.

all demonstrable lesions in the infected membranes; and the pathogenic effects of the virus so inoculated are little, if at all, surpassed by its direct cerebral inoculation.

It is not possible to detect the immediate effect of virus introduced directly into the brain, because of the injury of the tissues attending the injection. It is, however, common knowledge that monkeys which resist such inoculations, remaining free from obvious, detectable symptoms of disease, are as receptive to a subsequent injection of more potent virus as are control animals. Indeed, monkeys may resist more than one cerebral inoculation and yet respond to a later injection of virus in a manner indistinguishable from that exhibited by previously uninoculated animals. Since the injection of virus, once or repeatedly, into or beneath the skin in monkeys induces active immunity, the failure of a corresponding state to develop in intracerebrally inoculated animals is worthy of emphasis.

While it is, as stated, impossible, because of associated effects, to determine the immediate action of the virus when introduced cerebrally, no such barrier exists to the detection of effects when the virus is brought into contact with the olfactory area in the nasal membranes. If, for example, the cerebrospinal fluid is withdrawn by cisterna puncture and the cells present in it be counted, the striking fact will emerge that the presence of the virus invariably produces changes in the cell content, irrespective of whether symptoms of disease do or do not appear (3).<sup>2</sup>

This delicately adjusted response of the cerebrospinal fluid to the presence of the virus of poliomyelitis on the nasal membranes has engaged our attention for some time. We have followed the phenomenon under a variety of conditions and circumstances, which it will be the purpose of this paper and succeeding ones to describe. In studying the reaction we have had in mind its relation, if any, to the immunity which may be induced in monkeys by means of successive inoculations of the virus; and collaterally, the immunity which arises in children who are exposed to the virus and respond to its presence with mild attacks of illness—in their nature poliomyelitic—or who, through an unperceived or undetected series of events, become, in the

<sup>2</sup> It should be stated at this point that the instillation of sterile salt solution into the nares of *Macacus rhesus* on 6 successive days does not affect the number of cells in the cerebrospinal fluid withdrawn by cisterna puncture.

common phrase, specifically spontaneously immunized and protected against such infection.

This first paper will deal with the manner in which normal monkeys respond to one or multiple instillations of virus with changes in the cerebrospinal fluid, in comparison with discoverable alterations, of an immunity character, in the animals treated.

### *Methods*

*Instillation.*—1 cc. of a lightly centrifuged (300 revolutions per minute, for 1 minute) 10 per cent suspension of glycerolated monkey medulla and spinal cord, washed in two changes of salt solution, is instilled with a medicine dropper into each nostril of the animal.

For the second and for each subsequent nasal instillation, a new suspension is prepared, preferably from a glycerolated specimen other than that used previously. More consistent results can be obtained when suspensions are prepared from specimens which have been kept in glycerol less than 10 weeks.

The monkey is held by an assistant (no etherization is necessary for this procedure) in an upright position, with head bent backward. By attaching an ordinary rubber urethral tip to the dropper containing the virus, this device can be placed tightly against the nostrils and more force can be exerted, thus allowing the suspension to distribute itself to all parts of the upper nasal respiratory tract.

*Cisterna Puncture.*—The position of holding the monkey is important. After the animal has been thoroughly etherized, the back of the neck shaved and sterilized, an assistant places it on its abdomen, letting the head drop forward over the edge of the table; the assistant holds the head firmly with both hands.

A sharp hypodermic needle (1 inch cannula, No. 20 gauge) is inserted *vertically* almost its entire length until it reaches the cisterna magna region, which can be readily determined in the fingers by the sudden release of resistance. The stylet is then removed, and clear fluid is allowed to flow. The fluid is quickly taken off by a capillary pipette and placed in small Wassermann tubes.

Occasionally the first few drops of fluid may be tinged with blood, in which case pipetting is continued until a clear specimen is obtained.

*Cells.*—After gentle shaking, the cerebrospinal fluid is conveyed by a capillary pipette to the white cell counting chamber. No dye is added to the fluid. 4 square millimeters, or one large square in each corner of the usual 9 square millimeter cell counter, are counted each time.

If red cells are present, these can be eliminated by adding 3 per cent of an acetic acid solution, using equal parts of this solution and spinal fluid. A long pipette with a total capacity of 0.1 cc., divided into 100ths, is sufficiently accurate. The acetic fluid solution is drawn up to the 5th mark, after which the spinal fluid is brought up to the 10th or final mark. The entire contents are then expelled into a small, conical shaped test tube, allowing the mixture to remain in the tube for several minutes before counting.



## NASALLY INSTILLED VIRUS OF POLIOMYELITIS

*Globulin Test.*—The Noguchi butyric acid test brings out a positive reaction much earlier than do other methods.

(1) 0.5 cc. of a 10 per cent butyric acid solution, made up in salt solution, is added to 0.1 or 0.2 cc. of clear spinal fluid. The test tube is placed in boiling water and allowed to remain for 2 minutes in the boiling water bath.

(2) Then 0.1 cc. of N/10 sodium hydroxide is added and the tube is again placed in the boiling water for 1 or 2 minutes. Almost immediately, or in a few minutes, a precipitate forms and settles to the bottom in strongly positive reactions. If only a small amount of globulin is present, it may take up to an hour to form a precipitate.

*Temperatures.*—Rectal temperatures are taken daily, following the first instillation of virus and every day thereafter, usually in the morning, between 9:00 and 11:00 a.m. The thermometer is allowed to remain in the rectum for 1 minute. The Fahrenheit scale is used.

## PROTOCOLS

TABLE I  
*Macacus rhesus Responding Symptomatically to One Course of Instillations.*  
*Temperatures, Cell Counts, Globulin, and Symptoms*

| No. of monkey | Virus strain, dosage, dates of instillation                                     | Dates | Temperatures | Cells       | Globulin | Symptoms  |
|---------------|---|-------|--------------|-------------|----------|---|
| I             | Philadelphia, 1932, 4 doses, 3/27, 3/28, 4/5, 4/6/33<br><br>*Accelerating doses | 3/29  | 101.8        | 36 (normal) |          |   |
|               |   | 3/31  | 103          | 73          | +        |   |
|               |   | 4/3   | 103          | 96          | ±        |   |
|               |   | 4/5   | 102.6        | 108         | ±        |   |
|               |   | 4/6   | 102          | 193         | +        |   |
|               |   | 4/7   | 104.6        | 158         | +        | Tremor, ataxia; right deltoid paralyzed           |
|               |   | 4/8   | 104.6        | 166         | +        | No change   |
|               |   | 4/10  | 104.4        | 290         | +        | " "   |
|               |   | 4/11  | 105.4        |             | +        |   |
|               |   | 4/12  | 103          | 188         | ±?       | More active. Recovered without residual paralysis |
|               |   | 4/13  | 101          | 64          | ±?       |   |
|               |   |       |              |             |          |   |
|               |   |       |              |             |          |   |
|               |   |       |              |             |          |   |

\* The accelerating instillations affected temperatures, cell counts, and globulin content. They did not affect the paralytic symptoms.

TABLE I—*Concluded*

| No. of monkey | Virus strain, dosage, dates of instillation | Dates | Temperatures | Cells       | Globulin | Symptoms                                      |
|---------------|---|-------|--------------|-------------|----------|---|
| II            | Mixed, 2 doses, 6/14, 6/15/33               |       |              | 29 (normal) |          |   |
|               |   | 6/16  | 102.2        | 65          |          |   |
|               |   | 6/19  | 104.2        | 165         |          |   |
|               |   | 6/20  | 104.6        | 320         |          | Tremor  |
|               |   | 6/21  | 104.6        | 405         |          |   |
|               |   | 6/22  | 103.6        | 570         | +        | Ataxia, followed by paralysis, arms and legs  |
| III           | Havana, 1934, 6 doses, 1/2-1/8/35           |       |              | 22 (normal) |          |   |
|               |   | 1/9   | 103          | 36          |          |   |
|               |   | 1/11  | 104          | 148         |          |   |
|               |   | 1/14  | 105.6        | 496         |          | Deltoid paralysis, followed by prostration    |
| IV            | Mixed, 2 doses, 1/8, 1/10/35                |       |              | 36 (normal) |          |   |
|               |   | 1/12  | 103          | 140         |          |   |
|               |   | 1/14  | 105.2        | 504         |          |   |
|               |   | 1/16  | 103.4        | 580         | +        | Tremor, ataxia<br>Arms paralyzed<br>Prostrate |
|               |   | 1/17  |              |             |          |   |
| V             | Mixed, 3 doses, 1/8, 1/10, 1/12/35          |       | 103.6        | 29 (normal) |          |   |
|               |   | 1/14  | 103.6        | 490         |          |   |
|               |   | 1/16  | 106          | 610         | ++       | Ataxia<br>Arms paralyzed<br>Prostrate         |
|               |   | 1/17  |              |             |          |   |
| VI            | Philadelphia, 1932, 2 doses, 1/24, 1/26/35  |       |              | 30 (normal) |          |   |
|               |   | 1/26  | 101.2        | 35          |          |   |
|               |   | 1/28  | 105.2        | 374         |          |   |
|               |   | 1/30  | 103.2        | 580         | +        | Tremor, ataxia, arm paralysis                 |
| VII           | Cooperstown, 1928, 3 doses, 2/18-2/23/35    |       |              | 27 (normal) |          |   |
|               |   | 2/23  | 102.6        | 33          |          |   |
|               |   | 2/25  | 103.6        | 145         |          |   |
|               |   | 2/27  | 104.4        | 455         |          |   |
|               |   | 3/1   | 104.6        | 220         |          |   |
|               | Accelerating dose                           | 3/4   | 104          | 210         |          | Tremor<br>Deltoid paralysis                   |
|               |   | 3/6   | 103          | 365         |          |   |
|               |   | 3/8   | 104          | 390         |          |   |
|               |   | 3/12  | 102.8        | 405         |          | Arms, legs paralyzed                          |

TABLE II  
*Macacus rhesus* Responding Asymptomatically, with Cell Changes.  
 Temperatures, Cell Counts, Globulin, and Symptoms

| No. of monkey | Virus strain, dosage, dates of instillation | Dates   | Temperatures  | Cells  | Globulin | Symptoms                    |
|---------------|---|---|---|--|----------|-----------------------------|
| VIII          | New York, 1933, 2 doses, 12/26, 12/27/33    | 12/29<br>12/31<br>1/2/34<br>1/5                                     | 103.4<br>103.2<br>104.4<br>102  | 48 (normal)<br>97<br>120<br>410<br>640                                   |          | No symptoms                 |
| IX            | Mixed, 1 dose, 2/13/34                      | 2/14<br>2/15<br>2/16<br>2/17<br>2/19<br>2/20<br>2/21<br>2/24<br>3/5 | 103.4<br>103<br>103<br>103.2<br>104.4<br>104<br>102.4<br>102.8<br>101.8 | 42 (normal)<br>60<br>220<br>465<br>520<br>405<br>260<br>125<br>102<br>44 | 0        | No symptoms                 |
| X             | Mixed, 1 dose, 2/13/34                      | 2/14<br>2/15  | 103.4<br>102.2  | 55 (normal)<br>60<br>110   |          | (Sacrificed histology)* for |
| XI            | Mixed, 1 dose, 2/13/34                      | 2/14<br>2/15  | 101.8<br>104  | 35 (normal)<br>55<br>514   | ++       | (Sacrificed histology) for  |
| XII           | Philadelphia, 1932, 2 doses, 2/5, 2/7/35    | 2/7<br>2/9<br>2/11<br>2/13<br>2/16                                  | 103<br>103.2<br>103.4<br>103.6<br>102.4                                 | 14 (normal)<br>45<br>614<br>710<br>320<br>190                            |          | No symptoms                 |
| XIII          | Cooperstown, 1928, 4 doses, 2/18-2/23/35    | 2/23<br>2/25<br>2/27<br>3/1   | 102.6<br>103.6<br>104.4<br>104.6  | 27 (normal)<br>33<br>145<br>155<br>220                                   |          | No symptoms                 |

\* Two *Macacus rhesus* were injected as follows: one cerebrally and peritoneally with a suspension of the mixed medulla, pons and cervical cord; the other in the same manner, with a suspension of the intervertebral ganglia. No symptoms resulted in either animal.

TABLE III

*Macacus rhesus Given Two or More Courses of Instillations, the Earlier Nonsymptomatic, the Later Paralytic.  
Temperatures, Cell Counts, Globulin, and Symptoms*

| No. of monkey | Virus strain, dosage, dates of instillation          | Dates  | Temperatures | Cells       | Globulin | Symptoms  |
|---------------|--|--------|--------------|-------------|----------|---|
| XIV           | New York, 1933,<br>2 doses, 12/26,<br>12/27/33       | 12/26  | 102.6        | 48 (normal) |          | Fleeting symptoms,<br>including excitement and partial ptosis |
|               |  | 12/29  | 103.4        | 97          |          |   |
|               |  | 12/31  | 103.2        | 120         |          |   |
|               |  | 1/2/34 | 104.4        | 410         |          |   |
|               |  | 1/5    | 102          | 640         |          |   |
|               |  | 1/8    | 101.4        |             |          |   |
|               |  |        |              |             |          |   |
|               |  |        |              |             |          |   |
|               | New York, 1933,<br>6 doses, 2/5-<br>2/10/34          | 2/7    | 103          | 105         |          | No symptoms   |
|               |  | 2/9    | 103.4        | 62          |          |   |
|               |  | 2/13   | 102.6        | 100         |          |   |
|               |  | 2/15   | 102.8        | 45          |          |   |
|               |  | 2/16   | 101.6        |             |          |   |
|               |  |        |              |             |          |   |
|               | Philadelphia, 1932,<br>3 doses, 3/7, 3/9,<br>3/13/35 | 3/9    | 102.8        | 26 (normal) |          | No symptoms   |
|               |  | 3/11   | 103          | 24          |          |   |
|               |  | 3/13   | 101.8        | 65          |          |   |
|               |  | 3/15   | 103.6        | 34          |          |   |
|               |  | 3/18   | 103.6        | 64          |          |   |
|               |  |        | 101.6        | 43          |          |   |
|               | Havana, 1934, 3<br>doses, 5/15, 5/17,<br>5/21/35     | 5/18   | 103          | 31 (normal) |          | Tremor, ataxia, legs<br>paralyzed<br>Prostrate                |
|               |  | 5/21   | 103.2        | 33          |          |   |
|               |  | 5/22   | 105.6        | 37          |          |   |
|               |  | 5/24   | 105.6        | 265         |          |   |
|               |  |        | 106          | 580         | +        |   |
|               |  | 5/29   |              |             |          |   |
| XV            | New York, 1933,<br>2 doses, 12/26,<br>12/27/33       | 12/26  | 103          | 44 (normal) |          | No symptoms   |
|               |  | 12/29  | 103          | 58          |          |   |
|               |  | 12/31  | 102.6        | 230         |          |   |
|               |  | 1/2/34 | 103.6        | 260         |          |   |
|               |  | 1/5    | 103          | 365         |          |   |
|               |  | 1/8    | 102.4        |             |          |   |

\* The 1933 New York and the 1932 Philadelphia strains acted alike; the 1934 Havana strain acted differently. In keeping is the neutralization of 0.2 cc. Philadelphia virus by 0.8 cc. of serum taken after the Philadelphia virus instillations.

## NASALLY INSTILLED VIRUS OF POLIOMYELITIS

TABLE V  
*Macacus cynomolgus* Instilled Repeatedly and Tested for Antivirus

| Monkey   | Course | Virus strain, dosage, dates of instillation | Symptoms  |
|--|--------|---|---|
| <i>Cynomolgus</i><br>A<br><br>(Previously fed by stomach tube, without effect)<br><br>*Bled for serum, 3/10/32 | 1st    | Mixed, 6 doses, 11/2-11/7/31                | 6 days after the last instillation, deltoid became weak; no progression of paralysis. Complete recovery |
|  | 2nd    | Mixed, 6 doses, 1/4-1/9/32                  | No symptoms   |
|  | 3rd    | Mixed, 3 doses (accelerating), 1/16-1/18/32 | No symptoms   |
|  | 4th    | Mixed, 3 doses, 2/1-2/3/32                  | No symptoms   |
|  | 5th    | Mixed, 3 doses, 3/11-3/17/32                | No symptoms   |

*Neutralization Test*

\* Serum taken Mar. 10, 1932, after 4th course of instillation, mixed with virus in proportion of 0.9 cc. serum and 0.1 cc. filtrate of Mixed Virus; neutralized. Two injections given to 2 *rhesus* controls, Mar. 15 and 26, 1932 (acceleration); animals became paralyzed on 7th and 10th day respectively from same dose of virus filtrate.

*Intracerebral Test*

Apr. 22, 1932, 0.1 cc. Mixed Virus filtrate failed to induce symptoms. Two controls—one *cynomolgus* and one *rhesus*—became paralyzed on the 3rd and 8th day respectively from same dose of filtrate.

TABLE VI  
*Macacus cynomolgus* Instilled Repeatedly and Tested for Antivirus

| Monkey                 | Course | Virus strain, dosage, dates of instillation | Symptoms    |
|------------------------|--------|---|-------------|
| <i>Cynomolgus</i><br>B | 1st    | Mixed, 6 doses, 11/2-11/7/31                | No symptoms |
|                        | 2nd    | Mixed, 6 doses, 1/4-1/9/32                  | No symptoms |
|                        | 3rd    | Mixed, 3 doses (accelerating), 1/16-1/18/32 | No symptoms |
|                        | 4th    | Mixed, 3 doses, 2/1-2/3/32                  | No symptoms |
|                        | 5th    | Mixed, 6 doses, 3/11-3/17/32                | No symptoms |

\*Bled for serum, 3/10/32

*Neutralization Test*

\* Serum taken Mar. 10, 1932, after 4th course of instillations, mixed with Mixed Virus in proportion of 0.9 cc. serum and 0.1 cc. Mixed Virus filtrate, introduced into *Macacus rhesus*; no neutralization. Two injections of serum-virus mixture given: one, Mar. 15, 1932; the other (accelerating) on Mar. 26. Paralysis ensued on 10th day after the accelerating dose.

*Intracerebral Test*

Apr. 22, 1932, 0.1 cc. Mixed Virus filtrate injected into *Cynomolgus* B. Paralysis on 6th day. Two controls also became paralyzed.

TABLE VII  
*Macacus cynomolgus* and *Macacus rhesus* Instilled Repeatedly and Tested for Antivirus

| Monkey   | Course | Virus strain, dosage, dates of instillation | Symptoms           |
|--|--------|---|--------------------|
| <i>Cynomolgus</i><br>C<br>(Mate to tube-fed <i>Cynomolgus</i> A) | 1st    | Mixed, 6 doses, 11/2-11/7/31                | No symptoms        |
|  | 2nd    | Mixed, 6 doses, 1/4-1/9/32                  | Paralysis, 6th day |
| <i>Rhesus</i><br>D   | 1st    | Mixed, 6 doses, 1/4-1/9/32                  | No symptoms        |
|  | 2nd    | Mixed, 3 doses (accelerating), 1/16-1/18/32 | No symptoms        |
|  | 3rd    | Mixed, 3 doses, 2/1-2/3/32                  | No symptoms        |
|  | 4th    | Mixed, 3 doses, 3/11-3/17/32                | No symptoms        |

\*Bled for serum, 3/10/32

*Neutralization Test*

\* Mar. 15 and 26, 1932, (acceleration), 0.9 cc. serum and 0.1 cc. Mixed Virus filtrate into *Macacus rhesus*; no neutralization; paralysis 10th day after accelerating dose.

*Intracerebral Test*

Apr. 22, 1932, 0.1 cc. filtrate of Mixed Virus; paralysis 7th day.

In summing up the series of four monkeys (Tables V, VI, and VII) submitted to multiple courses of the instillation of virus, we find that, first, *Cynomolgus* A developed abortive symptoms of poliomyelitis from the first course, recovered, and proved durably immune, as shown by tests for antiviral and resistance to the cerebral injection of virus; second, that *Cynomolgus* B resisted five courses of instillation without producing detectable antiviral and without becoming in any degree refractory to a cerebral inoculation; and third, that *Rhesus* D behaved precisely as did *Cynomolgus* B, while *Cynomolgus* C resisted a first course of instillations merely to respond with paralysis to a second course given 2 months later.

Although these tests were carried out before the examination of the cerebrospinal fluid became a regular practice, there is every reason to believe that the succession of cell changes regularly occurred during the course of each series of instillations, as was always found to occur when examinations were made.

This series of tables completes the protocols of the main experiments. Tables VIII and IX which follow deal with complementary matters only. They are inserted to show: first, that the pleocytic cerebrospinal fluid contains no detectable virus at the height of the cell increases and even after acceleration inoculations; and, second, that repeated cisterna punctures in nonvirus-instilled animals do not change the average normal cell counts in the fluid.

TABLE VIII

*Macacus rhesus Injected Cerebrally with Pleocytic Cerebrospinal Fluid from Nasally Instilled Monkeys*

| Monkeys | Virus strain, dosage, dates of instillation | Cells   | Intracerebral inoculation                         | Symptoms    |
|---------|---|---|---|-------------|
| A       | Mixed, 2 doses, 6/14, 6/15/33               | 29 (normal)<br>6/16-6/19,<br>65-165<br>Fluid withdrawn each day and pooled    | 6/20/33, 1.6 cc., 1st pooled fluid                | No symptoms |
|         |   | 6/20-6/22/33,<br>320-570<br>Fluid withdrawn each day and pooled               | 6/28/33, 1.5 cc., 2nd pooled (accelerating) fluid | No symptoms |
| B       | Philadelphia, 1932, 2 doses, 6/14, 6/15/33  | 19 (normal)<br>6/16-6/19/33,<br>37-118<br>Fluid withdrawn each day and pooled | 6/20/33, 1.75 cc., 1st pooled fluid               | No symptoms |
|         |   | 6/20-6/22/33,<br>470-515<br>Fluid withdrawn each day and pooled               | 6/28/33, 2 cc., 2nd pooled fluid                  | No symptoms |

TABLE IX

*Normal Macacus rhesus Controls, Cisterna Puncture, with Cell Counts*

| Monkeys | June 21, 1933 | June 23, 1933 | June 26, 1933 | June 28, 1933 | June 30, 1933 |
|---------|---------------|---------------|---------------|---------------|---------------|
| C       | 22            | 26            | 20            | 35            | 25            |
| D       | 32            | 27            | 21            | 24            | 27            |



In summing up the series of four monkeys (Tables V: first, *Cynomolgus* A developed abortive symptoms of virus from the first course, recovered, and proved durably shown by tests for antiviral and resistance to the cerebral virus; second, that *Cynomolgus* B resisted five courses of without producing detectable antiviral and without becoming refractory to a cerebral inoculation; and third, that behaved precisely as did *Cynomolgus* B, while *Cynomolgus* a first course of instillations merely to respond with paralytic second course given 2 months later.

Although these tests were carried out before the examination of cerebrospinal fluid became a regular practice, there is every reason to believe that the succession of cell changes regularly occurred the course of each series of instillations, as was always found to be the case when examinations were made.

This series of tables completes the protocols of the main experiments in Tables VIII and IX which follow deal with complementary material only. They are inserted to show: first, that the pleocytosis of cerebrospinal fluid contains no detectable virus at the height of the increase and even after acceleration inoculations; and, second, that repeated cisterna punctures in nonvirus-instilled animals do not change the average normal cell counts in the fluid.

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TABLE VIII  
*Macacus rhesus* Injected Cerebrally with Pleocytic Cerebrospinal Fluid from Nasally  
 Instilled Monkeys

| Monkeys | Virus strain, dosage, dates of instillation | Cells  | Intracerebral inoculation                         | Symptoms    |
|---------|---|--|---|-------------|
| A       | Mixed, 2 doses, 6/14, 6/15/33               | 29 (normal)<br>6/16-6/19, 65-165<br>Fluid withdrawn each day and pooled    | 6/20/33, 1.6 cc., 1st pooled fluid                | No symptoms |
|         |   | 6/20-6/22/33, 320-570<br>Fluid withdrawn each day and pooled               | 6/28/33, 1.5 cc., 2nd pooled (accelerating) fluid | No symptoms |
| B       | Philadelphia, 1932, 2 doses, 6/14, 6/15/33  | 19 (normal)<br>6/16-6/19/33, 37-118<br>Fluid withdrawn each day and pooled | 6/20/33, 1.75 cc., 1st pooled fluid               | No symptoms |
|         |   | 6/20-6/22/33, 470-515<br>Fluid withdrawn each day and pooled               | 6/28/33, 2 cc., 2nd pooled fluid                  | No symptoms |

TABLE IX  
*Normal Macacus rhesus* Controls, Cisterna Puncture, with Cell Counts

| Monkeys | June 21, 1933 | June 23, 1933 | June 26, 1933 | June 28, 1933 | June 30, 1933 |
|---------|---------------|---------------|---------------|---------------|---------------|
| C       | 22            | 26            | 20            | 35            | 25            |
| D       | 32            | 27            | 21            | 24            | 27            |

In summing up the series of four monkeys (Tables V, VI, and VII) submitted to multiple courses of the instillation of virus, we find that, first, *Cynomolgus* A developed abortive symptoms of poliomyelitis from the first course, recovered, and proved durably immune, as shown by tests for antiviral and resistance to the cerebral injection of virus; second, that *Cynomolgus* B resisted five courses of instillation without producing detectable antiviral and without becoming in any degree refractory to a cerebral inoculation; and third, that *Rhesus* D behaved precisely as did *Cynomolgus* B, while *Cynomolgus* C resisted a first course of instillations merely to respond with paralysis to a second course given 2 months later.

Although these tests were carried out before the examination of the cerebrospinal fluid became a regular practice, there is every reason to believe that the succession of cell changes regularly occurred during the course of each series of instillations, as was always found to occur when examinations were made.

This series of tables completes the protocols of the main experiments. Tables VIII and IX which follow deal with complementary matters only. They are inserted to show: first, that the pleocytic cerebrospinal fluid contains no detectable virus at the height of the cell increases and even after acceleration inoculations; and, second, that repeated cisterna punctures in nonvirus-instilled animals do not change the average normal cell counts in the fluid.

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TABLE VIII  
*Macacus rhesus* Injected Cerebrally with Pleocytic Cerebrospinal Fluid from Nasally  
 Instilled Monkeys

| Monkeys | Virus strain, dosage, dates of instillation | Cells  | Intracerebral inoculation                         | Symptoms    |
|---------|---|--|---|-------------|
| A       | Mixed, 2 doses, 6/14, 6/15/33               | 29 (normal)<br>6/16-6/19, 65-165<br>Fluid withdrawn each day and pooled    | 6/20/33, 1.6 cc., 1st pooled fluid                | No symptoms |
|         |   | 6/20-6/22/33, 320-570<br>Fluid withdrawn each day and pooled               | 6/28/33, 1.5 cc., 2nd pooled (accelerating) fluid | No symptoms |
| B       | Philadelphia, 1932, 2 doses, 6/14, 6/15/33  | 19 (normal)<br>6/16-6/19/33, 37-118<br>Fluid withdrawn each day and pooled | 6/20/33, 1.75 cc., 1st pooled fluid               | No symptoms |
|         |   | 6/20-6/22/33, 470-515<br>Fluid withdrawn each day and pooled               | 6/28/33, 2 cc., 2nd pooled fluid                  | No symptoms |

TABLE IX  
*Normal Macacus rhesus* Controls, Cisterna Puncture, with Cell Counts

| Monkeys | June 21, 1933 | June 23, 1933 | June 26, 1933 | June 28, 1933 | June 30, 1933 |
|---------|---------------|---------------|---------------|---------------|---------------|
| C       | 22            | 26            | 20            | 35            | 25            |
| D       | 32            | 27            | 21            | 24            | 27            |

## DISCUSSION

The protocols presented establish several important points. In the first place, they show unmistakably that the bringing of the virus into contact with the nasal membranes is never an indifferent process in monkeys. The response to the presence of active virus is prompt and invariable; and this response takes place irrespective of whether obvious clinical signs of disease do or do not arise, and always in advance of any such symptoms as may arise.

Most monkeys do not resist the initial course of instillations; the response has, perhaps, little relation to the number of instillations in monkeys of average susceptibility. Certain monkeys, however, while not markedly refractory, possess a degree of resistance above the average; they respond to the larger number of instillations, and there is inconclusive evidence that the spacing of the instillations makes a difference. Occasionally, monkeys in which the cells in the cerebrospinal fluid, while increased, remain at a low level, will develop higher cell counts if the animals are reinstalled on the 8th to the 10th day, a procedure to which in instances of intracerebral reinoculation we have given the name "acceleration." Instilled monkeys which pass through the usual course of incubation, showing beginning and then severe symptoms of poliomyelitis, have rising cell counts, coinciding with rising temperatures, while those which escape obvious clinical signs of infection tend to have lower cell counts and a correspondingly lower temperature range. Globulin as an index of inflammatory changes in the cerebrospinal fluid is irregularly demonstrable and bears an inconstant relation to high cell count.

Among the refractory monkeys which have resisted the initial course of instillations (although exhibiting changes in the cerebrospinal fluid) are some which, after a rest period, come down characteristically in response to a second course of instillations of the same virus as that employed in the first course. The reason for this disparity is not known. There remains a small residue of monkeys which, having resisted two such courses, now seems capable of resisting multiple courses of instillation without developing obvious symptoms of infection. None of these exceptionally refractory monkeys is indifferent to the virus instillations, for all react with cell changes in the cerebro-

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spinal fluid. The refractory state, therefore, resides apparently in the nerve cells, the principal seat of usual virus attack—not in the nervous tissues as a whole.

Certain monkeys develop transient, slight, so called "abortive" symptoms of poliomyelitis as a result of the instillations. These animals have acquired increased resistance to virus injected into the brain, and their blood serum has become antiviral; and yet, reinstitution arouses cell changes in the cerebrospinal fluid qualitatively identical, quantitatively less marked, perhaps, than in monkeys failing to present obvious clinical signs of infection.

The series of reactions in the cerebrospinal fluid seems, therefore, independent of specific immune properties in the instilled monkeys. That there is no direct relationship between specific immunity and the changes induced in the cerebrospinal fluid is further shown by the important fact that, with present methods, active immunity invariably fails to develop in a detectable way, even in monkeys which have passed asymptotically through multiple courses of instillation. These animals which are highly refractory to nasal virus (although always, probably, exhibiting cerebrospinal fluid alteration), never develop humoral antiviral properties, and are as susceptible to the cerebral injection of virus as are the normal controls.

The last observation brings us back to a consideration of the relationship which may exist between the monkeys nasally instilled which develop no symptoms of illness, and the many instances of general immunity arising spontaneously in human populations from the chance entrance of virus into the nasal passages. All that can be stated at present is that the two species behave in diametrically opposite ways. While there is indubitable evidence that unperceived immunization is taking place widely among human populations, there is also evidence that monkeys, while subject to the direct inoculation of poliomyelitis, are strongly refractory to the virus in highly dilute condition, such as occurs in ordinary contact exposures; and this refractory state is bound up with the complete inability of the monkey to initiate the requisite physiological changes which attend and lead to active immunity, independent of symptomatic response, when the virus is introduced in a way to reach the central nervous organs directly.

## NASALLY INSTILLED VIRUS OF POLIOMYELITIS

There is, therefore, a close correlation discernible between the unsuccessful cerebral and the asymptomatic nasal inoculation of virus. Probably the directly injected virus which does not lead to symptoms, produces changes corresponding to those readily detectable in the cerebrospinal fluid of nasally instilled animals; and as such asymptomatic, cerebrally injected monkeys have not been rendered immune, so those failing to respond with symptoms to nasal inoculation, similarly acquire no immunity. From this it would appear that in their fundamental physiological reactions to the virus of poliomyelitis, man and the monkey are widely divergent.

The virus passing by way of the olfactory area of the nasal membrane to the brain acts on tissues directly, and not through intermediation of the cerebrospinal fluid. This fluid remains constantly free of detectable virus. Even at the earlier stages (Table VIII) no virus can be demonstrated in the fluid; and in the course of active disease, when nerve and supporting tissue cells are severely injured, virus appears never to escape in ascertainable quantities into the fluid, either in man or the monkey. The virus displays strong avidity for cells, in this respect exceeding in cellular affinity other viruses which attack the tissue structures of the nervous system.

It would not be without interest to ascertain whether, in the process of unperceived mass immunization of children, cell changes occur in the cerebrospinal fluid. We know already that such changes attend mild illnesses believed to be poliomyelitis; opportunity to determine this point will arise in connection with outbreaks in institutions. And we may learn that during epidemic prevalences of poliomyelitis, cases which hitherto have been diagnosed mild poliomyelitis, merely because pleocytosis has been discovered in the cerebrospinal fluid of anxious and nervous individuals, may be the objective index of an otherwise unperceived process of active immunization taking place within them.

## CONCLUSIONS

*Macacus rhesus* and *Macacus cynomolgus* exhibit a striking sensitivity to the presence of the virus of poliomyelitis on the nasal mucous membranes.

Irrespective of whether detectable symptoms of clinical poliomyelitis do or do not arise in the nasally instilled animals, the cere-

## FURTHER STUDIES ON THE SUBMAXILLARY GLAND VIRUSES OF RATS AND GUINEA PIGS

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PLATES 35 AND 36

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In a previous article, the occurrence of inclusion bodies in the submaxillary glands of wild rats, white stock mice, and hamsters, similar to those found in the salivary glands of guinea pigs and infants, was described (1). A virus specific for each of these three species of rodents, analogous to the submaxillary gland virus of guinea pigs (2), was demonstrated. The intracerebral injection of uninfected white rats, mice, or hamsters with emulsions of the homologous submaxillary glands showing characteristic pathological changes, usually killed the animals in 5-8 days. Histological examination of the brains showed a meningeal exudate consisting of mononuclear cells, some of which contained acidophilic intranuclear inclusion bodies. As in the case of the submaxillary gland virus of guinea pigs, in spite of the fact that the intracerebral inoculation almost invariably killed the animal, it was impossible to pass the mouse or hamster viruses from brain to brain. Each of these rodent submaxillary viruses proved to be specific: the hamster virus could not be transmitted to guinea pigs or mice, or the guinea pig virus to hamsters.

It was thought that further information in regard to these submaxillary gland viruses of rodents might be obtained by studying the wild rat virus in white rats. Although Thompson (3) previously reported the occurrence of inclusion bodies in the submaxillary glands of 2 months old rats, we have never observed a spontaneous infection in our stock white rats of any age. The wild rat virus would, therefore, be easier to work with than either the hamster or mouse virus, since in both hamsters and mice spontaneous infections are common.



with cerebrospinal fluid changes, differing only in degree from the nonimmune animals.

Detectable virus does not appear in the pleocytic cerebrospinal fluid at any stage of the pathological processes.

The current belief is that mass immunization is proceeding in an unperceived manner through the chance entrance of virus into the nasal passages of children. It is not known whether, apart from course of this unexpressed, cerebrospinal fluid changes occur in the to exist between man and the monkey in the capacity of the former to become immunized by way of the nasal membrane, and the inability of the latter to do so. It is common knowledge that monkeys do not become immune through unsuccessful cerebral inoculations of virus, and the same seems to be true of the nasal channel of virus penetration into the central nervous organs.

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rats withstood this dose of X-ray well, but many of the guinea pigs died following exposure.

In the case of the rats, the virus consisted of an emulsion of submaxillary glands showing the characteristic lesion, obtained from several full grown wild rats. In the case of the guinea pigs, an emulsion of the submaxillary glands of several full grown guinea pigs was used. Normal animals were inoculated at the same time as those exposed to X-ray. Under ether anesthesia, 0.1 cc. of the supernatant fluid obtained from the virus emulsion was inoculated intracerebrally into guinea pigs, and 0.05 cc. into rats. In the first generation no striking difference between the normal and X-rayed animals, either in severity of symptoms or duration of life was observed in guinea pigs or rats. In the first generation X-rayed animals were killed as soon as they appeared sick. After the removal of a piece of each brain for the preparation of histological sections, the rest of the brain tissue was emulsified and injected intracerebrally into other guinea pigs and rats which had been exposed to X-ray radiation. In a few instances it was possible to pass the guinea pig submaxillary gland virus and the rat submaxillary gland virus through 2 generations, but the inoculation of the brain emulsions of the second generation animals into other X-rayed animals gave negative results. Histological sections prepared from the brains of the X-rayed guinea pigs and rats did not show more extensive or striking lesions than those of the normal controls.

No evidence was obtained to indicate that the exposure to this dose of X-ray reduced the resistance of guinea pigs or rats sufficiently to make it possible to transmit these submaxillary gland viruses from brain to brain in series.

(b) *With the Addition of Testicular Extract (Duran-Reynals Factor).*—Testicular emulsions were prepared from the homologous species. Male rats and guinea pigs were killed and one testis was removed with sterile precautions. Each testis was cut up finely with scissors and emulsified by grinding with sand in 5 cc. of saline. Duran-Reynals suggests suspending the testicular material in approximately an equal volume of saline, but the suspension prepared in this way, proved too thick for intracerebral injection. After centrifugalization, the supernatant fluid was combined with an equal quantity of virus emulsion and injected intracerebrally into rats and guinea pigs. Control animals were injected with equal quantities of saline and virus emulsion.

No evidence was obtained to indicate that the Duran-Reynals factor (testicular extract) enhances the virulence of these two submaxillary gland viruses. It was impossible to transmit either the guinea pig or rat submaxillary gland virus from brain to brain with the addition of testicular extract.

(c) *Following Exposure to X-Ray and with Addition of Testicular Extract.*—In another series of animals the effect of X-ray and testicular extract were combined; equal quantities of virus emulsion and testicular extract were injected into rats and guinea pigs that had been exposed to 400 r units of X-ray. Attempts were made to carry the viruses through 5 generations in this way from brain to brain, transferring regularly on the 5th day. No evidence was obtained that the

## SUBMAXILLARY GLAND VIRUSES

Furthermore, it was thought possible that the wild rat virus might become more virulent by passage in the white rat.

The most striking characteristic of the guinea pig virus, which has also been shown to be true of the hamster and mouse viruses, is its marked predilection for the submaxillary gland. No matter where the virus is injected, it localizes in the salivary glands and produces typical pathological changes. It is only following intracerebral injection that it is possible to produce obvious symptoms or death, and then it is impossible to transmit the virus in series from brain to brain. Recently Hudson and Markham (4) have been able to carry the guinea pig submaxillary gland virus from brain to brain in one instance through 3 generations, and in another through 2. The virulence of the virus, however, decreased rather than increased, and it was impossible to continue intracerebral passage.

The intracerebral injection of the wild rat submaxillary gland virus into white rats produces symptoms of meningitis in the way characteristic of the other submaxillary gland viruses, but as with the guinea pig, hamster, and mouse viruses it has been impossible to transfer the rat virus from brain to brain in series. It was thought of interest to see whether serial intracerebral passage might be made possible either by reducing the resistance of white rats and guinea pigs by exposure to X-ray radiation, or by increasing the virulence of these two viruses by the addition of organ extracts (Duran-Reynals factor). Zinsser and Castaneda (5) have shown that the resistance of rats to typhus can be reduced by X-ray. Duran-Reynals (6, 7) has proved that certain organ extracts, particularly testicular extract, have the effect of enhancing the virulence of vaccinia. Hoffman (8) has confirmed Duran-Reynals' findings in regard to vaccinia, and has extended them to other viruses such as herpes, vesicular stomatitis of horses, and Borna disease.

*Attempts at Brain to Brain Transmission of the Virus*

(a) *Following Exposure to X-Ray.*—Young white rats approximately 6–8 weeks old and young guinea pigs, weighing about 180–220 gm. were exposed to 400 r units at kv. 160, ma. 8, filter 5.0 cm. oil, 0.25 mm. Al, (effective wave length 0.19 Å.u.) distance 50 cm., for 19 minutes 1–10 days before inoculation. This dose is somewhat less than that recommended by Zinsser and Castaneda. The animals, however, were very young, approximately 1–2 months old. The

rats withstood this dose of X-ray well, but many of the guinea pigs died following exposure.

In the case of the rats, the virus consisted of an emulsion of submaxillary glands showing the characteristic lesion, obtained from several full grown wild rats. In the case of the guinea pigs, an emulsion of the submaxillary glands of several full grown guinea pigs was used. Normal animals were inoculated at the same time as those exposed to X-ray. Under ether anesthesia, 0.1 cc. of the supernatant fluid obtained from the virus emulsion was inoculated intracerebrally into guinea pigs, and 0.05 cc. into rats. In the first generation no striking difference between the normal and X-rayed animals, either in severity of symptoms or duration of life was observed in guinea pigs or rats. In the first generation X-rayed animals were killed as soon as they appeared sick. After the removal of a piece of each brain for the preparation of histological sections, the rest of the brain tissue was emulsified and injected intracerebrally into other guinea pigs and rats which had been exposed to X-ray radiation. In a few instances it was possible to pass the guinea pig submaxillary gland virus and the rat submaxillary gland virus through 2 generations, but the inoculation of the brain emulsions of the second generation animals into other X-rayed animals gave negative results. Histological sections prepared from the brains of the X-rayed guinea pigs and rats did not show more extensive or striking lesions than those of the normal controls.

No evidence was obtained to indicate that the exposure to this dose of X-ray reduced the resistance of guinea pigs or rats sufficiently to make it possible to transmit these submaxillary gland viruses from brain to brain in series.

(b) *With the Addition of Testicular Extract (Duran-Reynals Factor).*—Testicular emulsions were prepared from the homologous species. Male rats and guinea pigs were killed and one testis was removed with sterile precautions. Each testis was cut up finely with scissors and emulsified by grinding with sand in 5 cc. of saline. Duran-Reynals suggests suspending the testicular material in approximately an equal volume of saline, but the suspension prepared in this way, proved too thick for intracerebral injection. After centrifugalization, the supernatant fluid was combined with an equal quantity of virus emulsion and injected intracerebrally into rats and guinea pigs. Control animals were injected with equal quantities of saline and virus emulsion.

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(c) *Following Exposure to X-Ray and with Addition of Testicular Extract.*—In another series of animals the effect of X-ray and testicular extract were combined; equal quantities of virus emulsion and testicular extract were injected into rats and guinea pigs that had been exposed to 400 r units of X-ray. Attempts were made to carry the virusts through 5 generations in this way from brain to brain, transferring regularly on the 5th day. No evidence was obtained that the

submaxillary gland viruses could be enhanced in virulence by these means. Two animals were injected in each transfer. Only the animals of the first generation appeared sick. In subsequent generations the animal not used for transfer, was observed for 2-3 weeks and then killed, and the submaxillary glands removed for histological examination. The glands of the 4th and 5th generations failed to show the specific lesion, indicating that by this method of rapid transfer from brain to brain, the virus was lost and failed to localize in the salivary glands in the usual way.

*Has Brain Tissue Any Inhibitory or Neutralizing Action on the Submaxillary Gland Viruses?*

In his original articles, Duran-Reynals (6, 7) compares the effect of other tissue extracts to testicular emulsions in enhancing the virulence of vaccinia. He found that kidney, liver, skin, and brain also had a slight tendency to increase the activity of vaccinia. On the other hand in the case of the submaxillary gland viruses, it seems possible that brain tissue might exert the opposite effect. In spite of the fact that animals injected intracerebrally with these viruses usually died, transfer from brain to brain in series was impossible. To test out the possible neutralizing action of brain tissue on the submaxillary gland viruses of guinea pigs and rats, the results of injecting equal quantities of submaxillary gland virus and brain emulsion, and equal quantities of submaxillary gland virus and testicular extract subcutaneously were compared. The subcutaneous inoculation of the submaxillary gland viruses, followed 2-3 weeks later by histological examination of the submaxillary glands for the presence of the specific lesion, is a more delicate method of testing for small amounts of these viruses than intracerebral injection.

*Method.*—In this instance, the rat virus was obtained from the submaxillary glands of 9 young rats which had been inoculated either subcutaneously or intracerebrally 2-4 weeks previously. Only one gland was obtained from each animal as the other one had been removed for histological section at the time of inoculation. Although as previously stated, no spontaneous infection in either young or full grown rats has been observed in this laboratory, nevertheless one of the submaxillary glands was usually removed as a control before any kind of injection was made. Whereas in the case of guinea pigs, hamsters, and mice it was necessary to use very young animals before they had become spontaneously infected, it was found that full grown white rats from our stock were as susceptible as young animals to intracerebral and subcutaneous injection. However, young animals be-

tween 1-2 months of age were used almost entirely, since it was thought that the chances of establishing the wild rat virus in them might be better than in adult animals.

A group of 6 young rats from each of which one submaxillary gland had been removed, was divided into 2 lots, 3 were inoculated subcutaneously with equal quantities of rat submaxillary gland virus and a freshly emulsified normal rat brain. The other 3 rats were injected subcutaneously with equal quantities of rat virus and freshly prepared rat testicular extract. The mixtures of rat virus and organ extracts were not allowed to stand, but were injected immediately. The animals were killed 2-3 weeks later, and the remaining submaxillary gland from all 6 animals was removed for the preparation of histological sections. All the submaxillary glands showed typical lesions and no differences were observed in the cellular reaction, or in the number of acidophilic intranuclear inclusion bodies, between the 2 lots of rats.

No evidence was obtained to indicate that extracts of brain tissue reduced the activity of the rat submaxillary gland virus, or that the addition of testicular extract produced any striking generalization of the virus following subcutaneous injection.

A similar experiment was carried out in guinea pigs with identical results.

#### *The Distribution of the Submaxillary Gland Viruses Following Intracerebral Injection*

The simplest explanation of the fact that it has been found impossible to date to transmit the submaxillary gland viruses from brain to brain in series, is that these viruses have no tendency to neurotropism. The invasion is limited to the meninges, and it is only when large doses are injected into the brain, that the local reaction is sufficiently intense to cause symptoms and death. If the animal survives the acute meningitis, the virus leaves the brain and invades the submaxillary glands. Even at the height of the meningeal reaction and in spite of the fact that inclusion bodies are numerous in the meningeal exudate, the virus does not seem to multiply to any great extent in the meninges. The inclusion bodies occur for the most part in large endothelial wandering cells. In the meninges of rats they are common in so called foreign body giant cells, such as may occur in many tissues in response to the injection of irritating, extraneous substances. It seems possible that these wandering cells may partially neutralize the activity of the

virus, so that when the brain of an animal dying of meningitis is injected intracerebrally into another animal, only a very slight reaction occurs, and the virus is soon lost on further passage from brain to brain. The virus is not, however, completely destroyed in animals dying of meningitis, since there is still sufficient virus in the brain emulsion to produce the specific lesion in the submaxillary gland of another animal when the infected brain tissue is injected subcutaneously.

The distribution of the virus was studied in a guinea pig showing symptoms of meningitis the 5th day following intracerebral inoculation of the guinea pig submaxillary virus.

The animal was killed and histological sections prepared from the brain and submaxillary glands. The brain showed a slight, localized meningeal reaction in which inclusion bodies were fairly numerous. The sections of the submaxillary glands failed to show any cellular reaction or specific lesion.

The brain, submaxillary glands, and one kidney were removed with sterile precautions and emulsified separately. Each of the 3 emulsions was tested for the presence of bacteria, and injected intracerebrally into 2 young guinea pigs. All 6 of the guinea pigs remained well and failed to show any appreciable symptoms. They were killed 16 days after inoculation, and the submaxillary glands removed for the preparation of sections. Only the glands of 2 animals that had been inoculated with the brain tissue showed small, very early lesions with typical intranuclear inclusion bodies. The glands from the 4 animals inoculated respectively with the submaxillary glands and kidney, proved negative.

This experiment indicates that the concentration of the virus in the brain of a guinea pig showing symptoms of meningitis 5 days after intracerebral injection, is too small to produce meningitis when injected into a second animal. The virus is, however, present in sufficient quantities to induce a localization of the virus in the submaxillary gland following subcutaneous injection. On the other hand the submaxillary gland and kidney did not in this instance contain enough virus either to produce meningitis when injected intracerebrally or to infect the submaxillary gland when injected subcutaneously.

In another experiment the distribution of the virus was studied in a rat inoculated intracerebrally with submaxillary gland virus on the 10th day following inoculation.

The animal was moribund and was killed with chloroform. Histological sections prepared from the brain showed a fairly extensive meningeal exudate con-

taining cells with acidophilic intranuclear inclusion bodies. Sections from the submaxillary glands showed a small early lesion with a few inclusion bodies. As in the previous experiment, the brain, submaxillary glands, and kidney were removed with sterile precautions, and emulsified separately. Each of the 3 emulsions was injected intracerebrally into 2 young rats. All 6 rats remained well and were killed on the 20th day after inoculation. The submaxillary glands of all 6 animals were removed for the preparation of histological sections. Slides from the glands of all the animals showed typical lesions with intranuclear acidophilic inclusion bodies in the duct cells.

This experiment indicates that when an animal lives as long as 10 days after intracerebral injection, the virus may be widely distributed in the body, but in none of the tissues was it in sufficient concentration to produce characteristic symptoms or death following intracerebral inoculation.

*Occurrence of the Virus in the Kidneys of Spontaneously Infected Animals in the Absence of Kidney Lesions*

The occurrence of the virus in the kidney of animals which had been inoculated intracerebrally, seemed to indicate either that the submaxillary gland virus might be eliminated through the kidney or that it might possibly localize in the epithelium of the kidney as well as in the submaxillary gland. Hindle and Stevenson (9) described the occurrence of intranuclear inclusion bodies in the kidney tubules of wild (London) rats. A spontaneous lesion in the kidneys of rats, guinea pigs, hamsters, or mice has not been observed in this laboratory. Nevertheless, it seemed of interest to try to determine whether there was any virus present in the kidneys of rats and guinea pigs with spontaneously infected submaxillary glands in the absence of demonstrable histological lesions.

The kidneys of several wild rats whose submaxillary glands were subsequently shown to contain typical lesions with acidophilic intranuclear inclusion bodies, were removed with precautions for asepsis. A piece of each kidney was put aside for the preparation of histological sections, and the rest of the kidneys were emulsified in the usual way. The emulsion was tested for the presence of bacteria, and then injected intracerebrally into 2 rats, and subcutaneously into 2 rats from which one submaxillary gland had been removed. The rats that had been inoculated into the brain, remained well. The animals that had been inoculated subcutaneously were killed 14 days after injection, and the remaining submaxillary



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gland removed for the preparation of histological sections. These sections showed typical lesions with acidophilic intranuclear inclusion bodies, whereas the glands removed from each animal before inoculation proved to be negative. No lesions were found in sections prepared from the kidneys used for inoculation.

In a similar experiment with guinea pigs, identical results were obtained. Although no demonstrable lesions were found in sections prepared from the kidneys of full grown guinea pigs with positive submaxillary glands, the subcutaneous injection of the kidney emulsion nevertheless produced characteristic changes in the submaxillary glands of young susceptible guinea pigs.

These experiments indicate that the submaxillary gland viruses of spontaneously infected wild rats, and full grown guinea pigs, are present in the kidney in spite of the lack of demonstrable pathological changes. The concentration of the viruses in the kidney seems to be much less than that in the submaxillary gland.

*Distribution of the Viruses in Rats and Guinea Pigs after Subcutaneous Injection*

In view of the findings stated above, it seemed of interest to determine how widely these viruses were distributed in the animal body following subcutaneous injection. A series of experiments was therefore done in young white rats and young guinea pigs to show in which tissues these viruses were present. The results in guinea pigs and rats were the same, so only one typical experiment will be cited.

The blood, cervical lymph nodes, submaxillary gland, spleen, liver, lung, and kidney were examined in the following way.

A young rat injected with virus subcutaneously 2 weeks before was anesthetized. 3-4 cc. of blood were obtained by cardiac puncture and placed in a sterile bottle containing sodium citrate. The animal was then killed, soaked in 5 per cent lysol, and the various organs removed with precautions for asepsis. Histological sections were prepared from each tissue. Pieces of the liver, lung, spleen, and kidney of approximately equal size were emulsified in the same quantity of saline. The amount of tissue derived from the submaxillary gland and cervical lymph nodes was smaller, but the same quantity of saline was added. All the tissues were ground with sand in the usual way. Cultures were made from each kind of tissue to rule out the presence of bacteria. 0.5 cc. of each organ emulsion and 0.5 cc. of citrated blood were injected subcutaneously into each of 3 rats, from which one submaxillary gland had been removed. The animals all remained well, and were

killed 2-3 weeks after inoculation. The remaining submaxillary gland was removed and histological sections prepared. No specific lesions were found in any of the tissues used for inoculation, except the submaxillary gland.

The submaxillary glands of the rats which had been injected with blood, liver, and spleen were uniformly negative in contrast to the submaxillary glands of the rats which had been injected with submaxillary gland, cervical lymph nodes, kidney, and lung which were usually positive. The lesions in the glands of the animals injected with submaxillary gland were nearly always the most extensive, and contained the greatest number of acidophilic intranuclear inclusion bodies. The concentration of the virus in the lung seemed very slight as judged by the fact that the inoculated animals were not uniformly positive and the lesions were always small. In order to obtain such a wide distribution, it is obvious that the blood of the animals which had been inoculated subcutaneously, must at some time have contained virus, but apparently these viruses do not persist in the circulation. In view of Thompson's recent observations (10) that intranuclear inclusions occur in the liver of apparently healthy rats, it is of interest that in these experiments no evidence was obtained of the virus being present in this organ.

#### *Direct Injection of the Viruses into the Kidneys of Rats and Guinea Pigs*

Since these viruses were found in the kidney of infected animals so frequently, it was thought possible that they might proliferate easily in the epithelium of the kidney following direct injection.

Direct injections of the homologous virus into the kidney of both rats and guinea pigs were made. The animals that survived operation, suffered no obvious ill effects from the injection, and were killed at various intervals. Often the scar made by the inoculation was visible in the gross. Sections were prepared both from the scarred area and from areas that appeared normal. The microscopic lesions produced by direct injection into the kidney were always circumscribed and more or less confined to the needle tract. The rest of the kidney did not become involved. The number of intranuclear inclusion bodies found was small and they were limited to the cells of the tubules in the area of scar formation (see Figs. 1 and 2). The glomeruli never contained inclusion bodies following direct injection into the kidney.

The marked predilection of these viruses for the submaxillary gland was also apparent in these experiments. Sections of the submaxillary glands of the animals inoculated directly into the kidney, nearly always showed definite lesions if the animals were killed 8 or more days after inoculation. The number of inclusion bodies in the submaxillary gland was consistently greater than that found in the kidney itself.

In this connection it is of interest to note that on 2 occasions in guinea pigs which had been exposed to X-ray and which had reacted severely to radiation, microscopic lesions were found in the kidney as well as in the submaxillary gland as the result of subcutaneous injection of the guinea pig submaxillary gland virus. In one of these guinea pigs the lesion in the kidney was fairly extensive: typical acidophilic intranuclear inclusion bodies were present in the cells of tubules, in the interstitial tissue, and in the glomeruli (Fig. 3). In this animal, the site of inoculation was hemorrhagic and necrotic. Sections prepared from this area showed typical inclusion bodies in the subcutaneous tissue (Fig. 4). A local reaction of this kind was never obtained in normal guinea pigs following subcutaneous injection.

The subcutaneous injection of the rat submaxillary gland virus into rats that had been exposed to a similar dose of X-ray did not produce any gross local reaction. As stated before, young rats seemed consistently more resistant to X-ray than young guinea pigs.

#### *Can "Virus" Pneumonia Be Produced with the Submaxillary Gland Viruses?*

In recent years the concept (11) that certain epidemic diseases presumably due to filtrable viruses, such as measles, epidemic influenza, and whooping cough, are often complicated by a characteristic type of pneumonia, has been accepted by many workers. This so-called virus pneumonia differs from the usual pyogenic lobar or lobular pneumonia by certain well defined pathological findings. It is essentially an interstitial pneumonia in which the more acute stages are characterized by edema and hemorrhage into the alveoli. The alveolar walls tend to become thickened by the invasion of mononuclear cells of various kinds. Whatever purulent exudate is present, is in most cases confined to the bronchi. There is a marked thickening of

the bronchial walls due to the presence of a "collar" of mononuclear cells. These collars are sometimes so conspicuous that they can be observed with the naked eye.

Several observers (12-14) have recently emphasized the occurrence of this type of pneumonia in children dying of pertussis. In addition to finding pathological changes consistent with a virus pneumonia, acidophilic intranuclear inclusion bodies have been found in a fairly high proportion of the cases coming to autopsy. In order to evaluate the significance of the inclusion bodies occurring in pertussis, McCordock and Smith (14) have also studied the incidence of intranuclear inclusion bodies in the submaxillary glands of children dying of this disease. These authors are of the opinion that inclusion bodies occur more frequently in the submaxillary glands of children dying of whooping cough, than in the submaxillary glands of children dying from other causes. McCordock and Smith raise the question whether the inclusion bodies found in the lungs of pertussis cases, could be due to the activity of a filtrable virus present in the salivary glands, or to a specific filtrable virus which is the cause of whooping cough.

In a previous paper (1) one of us attempted unsuccessfully to demonstrate a filtrable virus in the submaxillary glands of children dying from miscellaneous causes, which showed typical acidophilic intranuclear inclusion bodies. In spite of the fact that no infectious agent has to date been shown to be present in the salivary glands of children which show these changes, it seemed of interest to study the effect of intratracheal injections of the submaxillary gland viruses of rats and guinea pigs.

It is often considered that virus pneumonia as it occurs in man, is the result of the combined action of a virus and bacteria. The virus is thought to facilitate the entrance of the bacteria into the lung. Shope (15) has conclusively proved the dual etiology of swine influenza.

*Methods.*—In these experiments, the animals were divided into 4 groups: one received virus alone, one virus and bacteria, one heat-killed virus, and one bacteria alone. The methods used in guinea pigs and rats were essentially the same. The virus was prepared in the usual way from the submaxillary glands of animals that had been injected with virus subcutaneously 2 or more weeks previously. In order to estimate the potency of the particular lot of virus used, intracerebral injections were made at the same time as the intratracheal. The viruses were killed by heating at 60°C. for 30 minutes.

The marked predilection of these viruses for the submaxillary gland was also apparent in these experiments. Sections of the submaxillary glands of the animals inoculated directly into the kidney, nearly always showed definite lesions if the animals were killed 8 or more days after inoculation. The number of inclusion bodies in the submaxillary gland was consistently greater than that found in the kidney itself.

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The submaxillary glands of both rats showed areas of cellular infiltration, and in the gland of one of the animals typical inclusion bodies were found.

Some of the animals were killed at longer intervals, 12-14 days after injection. The lesions in both rats and guinea pigs were less striking than those in animals killed after 7-8 days, although a few inclusion bodies were found in the alveoli.

Inclusion bodies were never found in the lungs of the animals that had been injected either with heat killed virus, or with the strain of rat influenza bacilli.

Intratracheal injection of the virus in almost every instance led to a rapid invasion of the submaxillary gland. Sections from the submaxillary glands of nearly all the animals surviving 7 days or longer that had been injected with living virus were positive. The glands of the animals that received either heat-killed virus or bacteria alone were uniformly negative.

These experiments indicate that it is possible to produce the picture of virus pneumonia by intratracheal inoculation of the submaxillary gland viruses in rats and guinea pigs provided large doses of virus are injected. The results obtained are similar to those of Muckenfuss *et al.* following intratracheal injection of vaccine virus into rabbits (16). No evidence was obtained that these viruses facilitated the invasion of the strain of rat influenza bacilli used. The injection of foreign material of any sort intratracheally produces considerable reaction. These viruses show a much greater tendency to proliferate in the submaxillary gland than in lung tissue.

#### *Does Transfer in Young Rats Increase Virulence?*

Stewart and Duran-Reynals (17) have shown that vaccine virus tends to become generalized following intradermal injection when combined with testicular extract.

The wild rat virus was transferred subcutaneously at regular intervals of 10-14 days, with and without the addition of testicular extract through 6 generations of young white rats. All the animals remained well, and the virus localized in the submaxillary glands in the usual way. No evidence was obtained to indicate that the virus increased in virulence.

#### *Injection of the Wild Rat Virus Intratesticularly*

The wild rat virus was injected directly into the testis of young white rats. The injected testis showed no definite local reaction. Sections prepared from the testis showed small areas of cellular reac-

*Bacteria*.—A strain of small Gram-negative bacilli closely resembling human influenza bacilli was isolated from the throat of a normal rat. These organisms grew more luxuriantly on "chocolate" agar than on blood agar, but failed to grow on plain agar. The same culture was used for guinea pigs and rats and will hereafter be designated as the rat "influenza" strain. Several chocolate agar slants were washed off with saline and a moderately heavy suspension was used.

The animals to be injected were anesthetized, and one submaxillary gland removed for the preparation of histological sections. At the same time the trachea was exposed and a direct injection made, 0.3–0.5 cc. into rats and 0.7–1 cc. into guinea pigs. The animals injected with the mixture of virus and bacteria, received equal quantities of virus emulsion and bacterial suspension, the animals receiving virus alone, an equal quantity of virus and saline. Irrespective of what had been inoculated, many of the animals died shortly after injection. The animals that died soon showed in the gross extensive hemorrhages in the lungs. Microscopically, the main findings were hemorrhage and edema into the alveoli, and purulent exudate in the bronchi.

Most of the animals that survived for several days did not appear to be sick. One guinea pig which had received virus alone was found dead on the 8th day. The lungs of this animal appeared slightly hemorrhagic at autopsy, but no definite consolidation was apparent. Microscopically, a moderately extensive interstitial pneumonia was present, and many of the alveolar and wandering endothelial cells contained acidophilic intranuclear inclusion bodies (Figs. 5 and 6). The trachea also showed areas of cellular reaction in the interstitial tissue and intranuclear acidophilic inclusion bodies were found in large mononuclear endothelial cells. The tracheal epithelium itself was never involved. Sections of the submaxillary gland showed a very small area of reaction in which two typical small intranuclear inclusion bodies were found. No well defined bronchial collars were observed in this animal.

Two rats, one injected with virus alone, and one with virus and bacteria, were killed 7 days after inoculation, and sections prepared from the lungs and the remaining submaxillary glands. In the gross the lungs appeared more or less normal except for one small area of hemorrhage in the rat injected with virus and bacteria. Microscopically, the lesions in the animal receiving virus and bacteria were more extensive than those in the animal receiving virus alone, and there was a marked purulent bronchial exudate present. Both these animals showed a small number of well marked collars around the bronchi consisting mainly of large, wandering mononuclear cells, some of which contained typical intranuclear inclusion bodies (Figs. 7 and 8). The trachea of both these rats showed areas of cellular reaction in the interstitial tissue. Acidophilic intranuclear inclusion bodies were present in large mononuclear endothelial cells (Fig. 9). The tracheal epithelium itself was not involved. In the rat that received the mixture of virus and rat influenza bacilli no masses of organisms, such as are commonly seen in children dying of pertussis, were found amidst the cilia of the tracheal epithelium.

One of the important characteristics of certain filtrable viruses is their tendency to invade the nervous system. In many diseases in which a virus etiology is proven or suspected, *e.g.* vaccinia, variola, measles, and pertussis, encephalitis is fairly common. This aspect of filtrable viruses has been intensively studied in recent years. It is worthy of note that in mumps, an infectious disease of the salivary glands, meningitis is not an infrequent complication. Johnson and Goodpasture (19) have been able to transmit mumps to monkeys, but no inclusion bodies have been found in the infected submaxillary glands of these animals. To date no work has been reported as to the effect of intracerebral or intraspinal injection of the mumps virus into monkeys.

Although the submaxillary gland viruses of rodents, when injected intracerebrally, produce a characteristic type of meningitis, it has been impossible to modify them in such a way as to make them attack the nervous tissue itself, either by the addition of the Duran-Reynals factor, or by reducing the resistance of the host by X-ray.

The distribution of the submaxillary gland viruses in the body of spontaneously and artificially infected animals seems to be fairly wide. The viruses have been found in tissues (kidney and lung) in which no pathological evidence of their presence is demonstrable. They are apparently disseminated *via* the circulation, but do not seem to persist for any length of time in the blood. Attempts to adapt the submaxillary gland viruses of rats and guinea pigs to other organs such as the kidney, lung, and testis, have not met with very striking results. Whether or not the acidophilic intranuclear inclusion bodies reported by Findlay (20) in the livers of Clacton mice, and in the livers of both mice and rats by Thompson (10), are due to the submaxillary gland viruses, or some other viruses, remains undetermined. In our experiments following subcutaneous injection, the liver apparently did not tend to harbor the virus.

The submaxillary gland viruses as they occur in guinea pigs, hamsters, mice, and rats are remarkably uniform in their properties, and no significant variations have been observed. Cowdry (21) has recently reported the occurrence of acidophilic intranuclear inclusion bodies in the submaxillary glands of a certain species of monkey (*Cebus fatuellus* L.). It would be of interest to determine the presence of a virus in this higher form, and compare it to the rodent viruses.



tion, but no intranuclear inclusion bodies were found either in the interstitial or parenchymal cells. Sections prepared from the submaxillary glands of the animals 2 or more weeks after intratesticular injection, usually showed characteristic lesions.

*Does Generalization Occur if the Salivary Glands Have Been Removed?*

The predilection of the submaxillary gland virus for the salivary glands is so characteristic that it seemed of interest to try to determine how the rats would take care of the virus if these glands were removed as completely as possible. Under ether anesthesia, all the recognizable salivary gland tissue and cervical lymph nodes were excised. After 1 or 2 days when the animals had recovered from the operation, the rats were injected subcutaneously with virus emulsion. The animals remained well, and were killed after 2-3 weeks. At autopsy a small fragment of salivary gland could always be found, which showed typical lesions.

As in the case of guinea pigs, it was found that total extirpation of the salivary gland tissue was impossible.

*Do the Lesions in the Salivary Glands of Infants Persist to Adult Life?*

In rodents the lesions in the salivary glands, usually acquired in the first few months of life, seem to persist indefinitely. No one to date has described lesions in the salivary glands of human adults similar to those found in infants. In one instance VonGlahn and Pappenheimer (18) have described visceral lesions such as occur in infants, in a man 36 years of age.

It seemed of interest to study sections of surgical specimens containing fragments of more or less normal submaxillary gland removed during the course of various operations. About 25 specimens of this kind have been examined, but no inclusion bodies were found.

#### DISCUSSION

The submaxillary gland viruses as they occur in rodents represent a benign infection. The most striking characteristic of these viruses, as stated before, is their marked predilection for the salivary glands. Once infected, the animals harbor the infectious agent throughout life, and the virus can be isolated from salivary glands that show the characteristic lesions at any time, although the host himself is immune.

One of the important characteristics of certain filtrable viruses is their tendency to invade the nervous system. In many diseases in which a virus etiology is proven or suspected, *e.g.* vaccinia, variola, measles, and pertussis, encephalitis is fairly common. This aspect of filtrable viruses has been intensively studied in recent years. It is worthy of note that in mumps, an infectious disease of the salivary glands, meningitis is not an infrequent complication. Johnson and Goodpasture (19) have been able to transmit mumps to monkeys, but no inclusion bodies have been found in the infected submaxillary glands of these animals. To date no work has been reported as to the effect of intracerebral or intraspinal injection of the mumps virus into monkeys.

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## CONCLUSIONS

1. It has not been possible to increase the virulence of the submaxillary gland viruses of guinea pigs and rats, either by reducing the resistance of the animals by exposure to X-ray, or by the addition of testicular extract (Duran-Reynals factor).

2. In guinea pigs and wild rats with spontaneously infected submaxillary glands, the kidney has been found to contain the virus in the absence of demonstrable pathological changes.

3. Direct injection of these viruses into the kidney produces only mild, circumscribed lesions.

4. The viruses, following subcutaneous injection into white rats and guinea pigs, are widely distributed 2 weeks after injection. They are present in the submaxillary glands, cervical lymph nodes, kidney, and lung. They were not demonstrable at this time in the blood, liver, or spleen.

5. By the intratracheal injection of large doses of virus in guinea pigs and rats, an interstitial bronchopneumonia with thickening of the alveolar and bronchial walls and the presence of acidophilic inclusion bodies, can be produced.

6. No evidence was obtained to indicate that the multiplication of bacteria in the lung is greatly enhanced by the injection of these viruses.

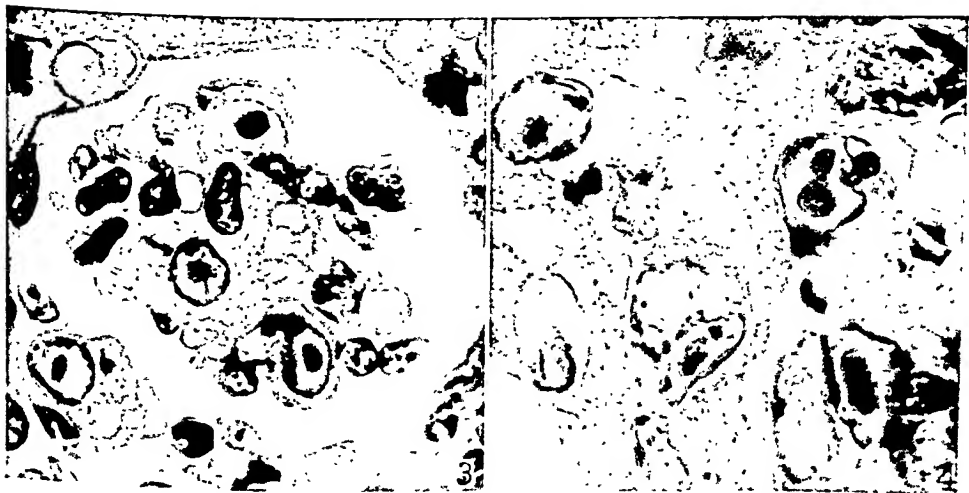
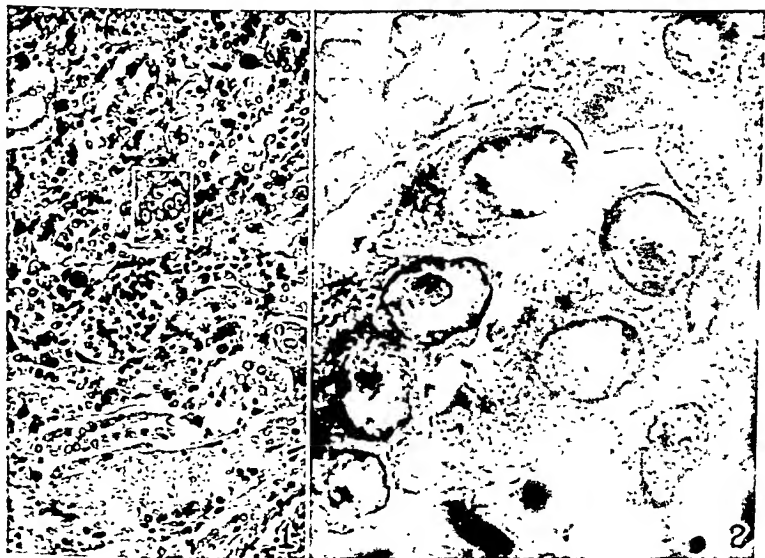
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In the case of man, the virus etiology of the lesions found in the salivary glands of infants has yet to be proved. However, certain differences in the pathological changes as they occur in man and in rodents are apparent at the present time. In rodents it has been emphasized that new-born animals are uniformly free of the infection, and there is no evidence indicating intra-uterine infection. In man, on the other hand, as previously pointed out (1), although lesions in the submaxillary glands of infants have not been reported before the age 2 months, visceral lesions with hypertrophied cells and typical acidophilic intranuclear inclusion bodies have been found in still-births and infants that lived only 1-2 days. Of course it may be that we are dealing with 2 different viruses, but if it is a virus at all, we have to assume that the still-births were infected *in utero*.

The other interesting difference between man and rodents in respect to these pathological changes in the salivary glands, is the tendency for these lesions to persist in full grown animals, whereas in human beings as far as we know, they have never been reported in adults. On one occasion (18) visceral lesions similar to those of infants have been described in a man of 36 years.

The most important aspect of the submaxillary gland viruses is the one emphasized by the work of McCordock and Smith (14): Provided that the lesions in the salivary glands of infants are due to an infectious agent, can this virus under certain circumstances invade the lung? Although it is always dangerous to draw analogies from one animal species to another, it seemed of interest to determine what kind of lesions resulted from the intratracheal injection of the submaxillary gland viruses of rats and guinea pigs. By the injection of large quantities of virus, pathological changes suggestive of a virus pneumonia could be produced in the lungs of guinea pigs and rats. These viruses, however, showed no marked tendency to invade lung tissue, and even when mixtures of virus and bacteria were injected intratracheally, no severe lesions developed. These viruses did not seem to facilitate the multiplication of bacteria in the lung. The virus in salivary glands of man, however, may be more virulent than that of rodents. Perhaps further light can be thrown on this question by studies in the monkey (*Cebus fatuellus* L.).



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#### EXPLANATION OF PLATES

##### PLATE 35

FIG. 1. The kidney of a guinea pig injected directly with the guinea pig submaxillary gland virus 8 days after injection. Inclusion bodies are visible in the tubule just above the glomerulus.  $\times 155$ .

FIG. 2. The same tubule under high power showing several small inclusion bodies.  $\times 1290$ .

FIG. 3. Inclusion bodies in the glomerulus of an X-rayed guinea pig inoculated with virus subcutaneously, 9 days after injection.  $\times 1145$ .

FIG. 4. Inclusion bodies in the subcutaneous tissues at the site of inoculation of the same guinea pig as Fig. 3.  $\times 1145$ .

##### PLATE 36

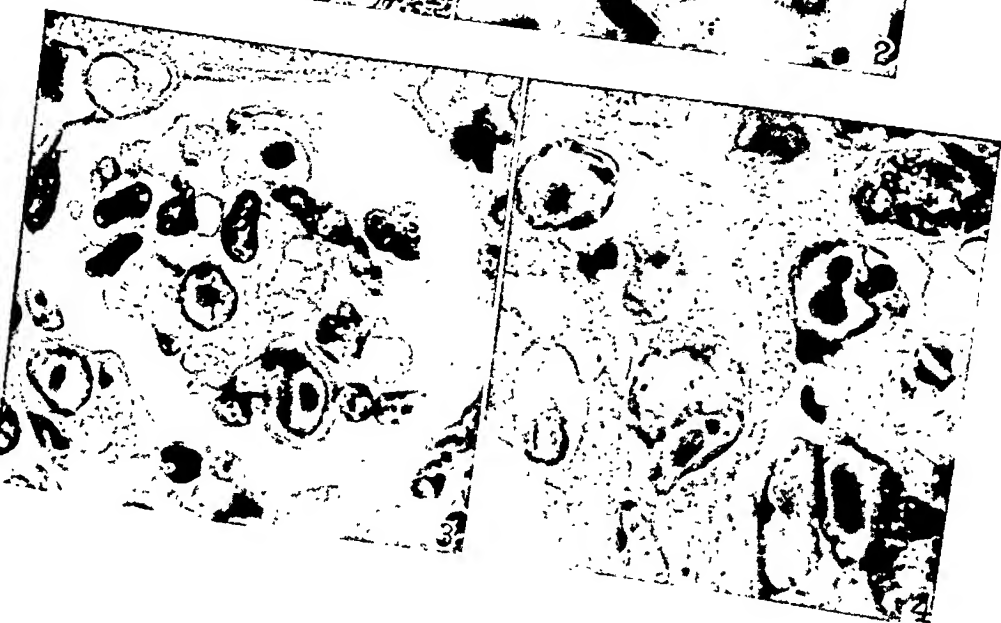
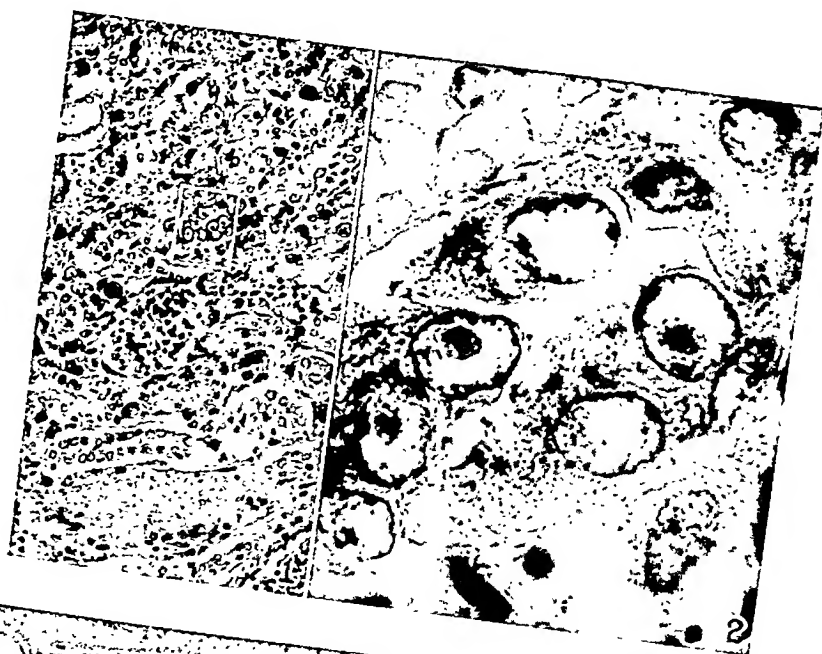
FIG. 5. The lung of a guinea pig found dead 8 days after intratracheal injection of guinea pig submaxillary gland virus, showing hemorrhage and thickening of the alveolar wall.  $\times 145$ .

FIG. 6. High power of Fig. 5 showing an intranuclear inclusion body in the alveolar wall.  $\times 1210$ .

FIG. 7. The lung of a rat 7 days after the intratracheal injection of the rat submaxillary gland virus and bacteria showing the thickening of the bronchial wall. Several inclusion bodies are visible in the collar surrounding the bronchus.  $\times 145$ .

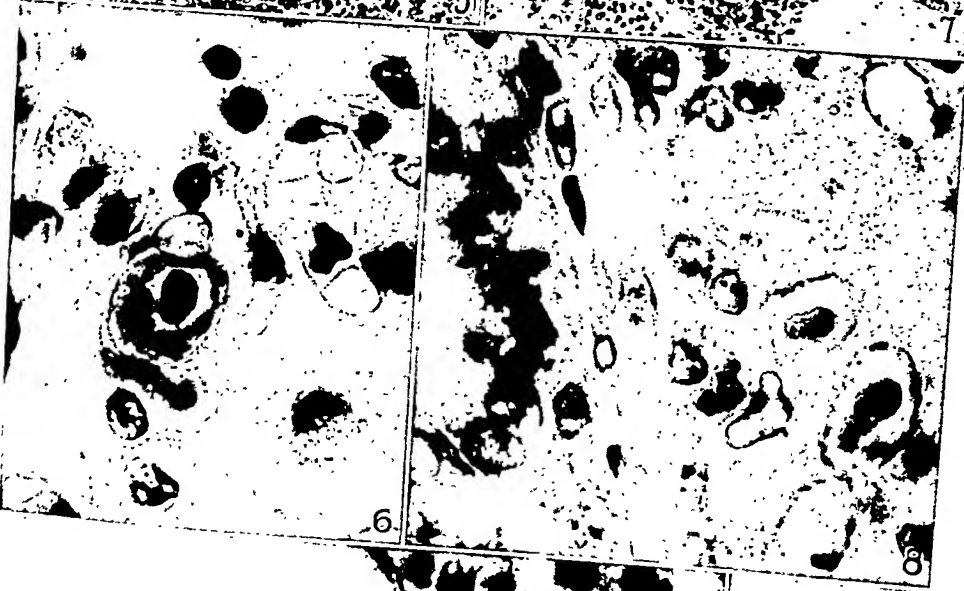
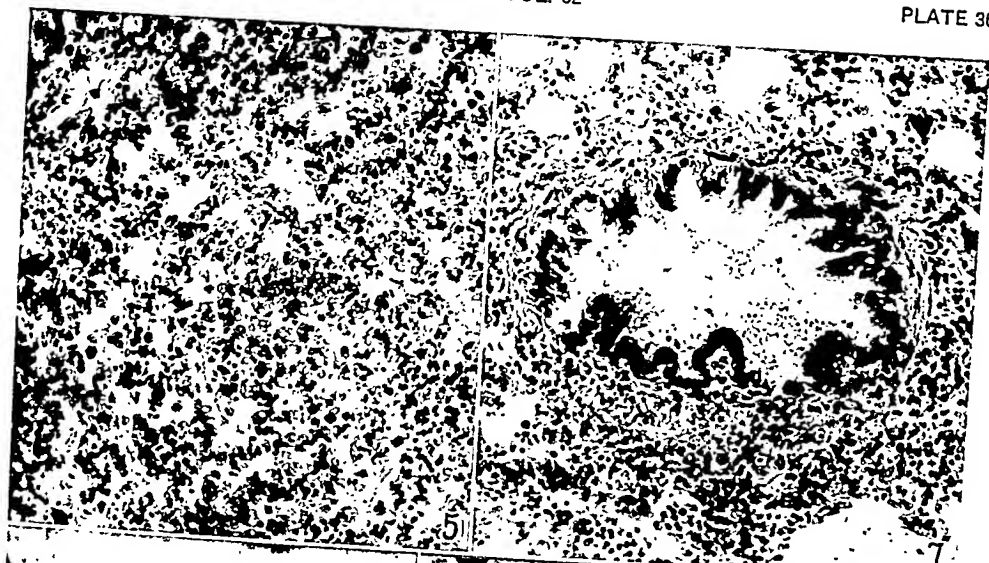
FIG. 8. High power of Fig. 7 showing inclusion bodies.  $\times 1210$ .

FIG. 9. The trachea of the same rat as Fig. 8 showing inclusion bodies in 2 endothelial cells lying just below the tracheal epithelium.  $\times 1210$ .











## THE DISTRIBUTION OF SWINE INFLUENZA VIRUS IN SWINE

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(Received for publication, September 19, 1935)

Swine influenza is a disease of complex etiology. It is caused by the concerted action of a filtrable virus and the bacterium, *Hemophilus influenzae suis*; and neither agent alone is capable of inducing the disease (1).

While the virus of swine influenza has been found regularly in mixtures of lung and bronchial lymph nodes from infected pigs (1 and 2), it was not demonstrated, in a small number of earlier unpublished experiments, in either the spleen or heart blood by swine inoculation. Furthermore the virus was innocuous when administered intramuscularly to swine (3) and produced influenza only when it gained entrance by way of the respiratory tract. These facts are in accord with Waldmann's conception of it as a pneumotropic virus (4).

However, certain features of the clinical and pathological pictures exhibited by swine suffering an acute influenza suggest a generalized or septicemic infection rather than one in which the etiological agents (1) are limited to the respiratory tract. The prostration of infected swine is more extreme than might be expected from the amount of pneumonia encountered at autopsy, and the accompanying leukopenia (2) suggests the possibility of the presence of the infectious agents in the blood stream. At autopsy pathological alterations are encountered outside the respiratory tract: The cervical, bronchial and mesenteric lymph nodes are frequently enlarged and edematous, the spleen is usually swollen and engorged and the mucosa of the colon is congested and sometimes edematous (2). It was known from bacteriological studies that, except in fatal cases, *H. influenzae suis* was seldom encountered outside the respiratory tracts of swine ill of influenza (5). This organism could thus not be held directly accountable and it seemed possible that the filtrable virus might be responsible for the features of swine influenza suggesting a generalized infection.

The experiments reported in the present paper were conducted in an effort to determine whether the swine influenza virus was limited strictly to the respiratory tract or whether it became generalized during the course of the disease. The observation by Andrewes, Laidlaw and Smith (6) that the swine influenza virus is pathogenic for white mice has been utilized in this study of the distribution of virus in influenza-infected swine.

#### EXPERIMENTAL

The 8 swine used in the present experiments were infected by intranasal inoculation with glycerolated swine influenza virus mixed with a small amount of a culture of *H. influenzae suis* (2). All developed typical swine influenza and were killed with chloroform on either the 3rd or 4th day following inoculation. Portions of the organs to be tested for the presence of virus were removed at autopsy with sterile instruments. They were then ground with sand and physiological salt solution was added to make an approximately 5 per cent suspension. The suspensions were allowed to sediment for 10 minutes before the supernatant fluid was decanted to be used in inoculating mice. The tracheal exudate was scraped from the opened trachea with a sterile spatula and was prepared as an approximately 5 per cent suspension by shaking in a flask containing glass beads and physiological saline. Blood was obtained from the swine at autopsy by pipette from the seared heart. It was defibrinated with a wire whip and used undiluted in inoculating the test mice.

The white mice used in testing for the presence of virus in the various organ suspensions were inoculated intranasally, while under ether narcosis, as previously described (7). They were kept under observation for 4 days and the survivors were then chloroformed and examined for the presence of the characteristic pneumonia caused by the swine influenza virus (6 and 7). Some of the mice, especially those receiving tracheal exudate and lung, succumbed to the swine influenza virus infection on the 3rd or 4th day. Three mice were inoculated with each suspension in most cases. The results are summarized in Table I.

#### DISCUSSION

As shown by the data in Table I, swine influenza virus was present in the lungs, tracheal exudate and turbinates of all swine tested. It was demonstrated in only two instances in tissues outside the respiratory tract. One of each group of 3 mice inoculated with suspensions of the bronchial lymph nodes of Swine 1539 and Swine 1574 developed a scant influenzal pneumonia. To be certain that the lesions in these 2 mice were due to swine influenza virus their lungs were used to infect other mice in series; these mice all developed typical and extensive



influenzal pneumonias. The bronchial lymph nodes of 2 of the 8 swine examined thus contained swine influenza virus but, as judged by mouse inoculation, in very low concentration. No virus was detected in the spleens, livers, kidneys, mesenteric lymph nodes, colon mucosae, brains or blood of any of the swine studied.

Mice inoculated intranasally with either the fresh defibrinated swine blood or swine serum exhibited a picture at postmortem which deserves special comment. Their lungs contained pale grey areas of consolidation that were similar in distribution to the lesions caused by the swine influenza virus. The possibility was at first entertained that these pneumonic areas might represent unusual virus reactions. However, all attempts to transmit virus serially in mice from such lesions were unsuccessful, the lungs of mice of the first serial transfer proving normal at autopsy.

No evidence was obtained to indicate that the swine influenza virus was generally distributed throughout the bodies of any of the 8 swine studied. It was confined to the respiratory tracts of 6 of the animals and in the remaining 2 to the respiratory tracts and the regional lymph nodes. The virus evidently has a strong affinity for the respiratory tract and exerts its specific effect there. Those features of swine influenza suggesting a generalized or septicemic infection appear, therefore, to be secondary effects of the localized respiratory tract disease.

#### SUMMARY

Swine influenza virus was found to be regularly present in the turbinates, tracheal exudate and lungs of infected swine but not in the spleens, livers, kidneys, mesenteric lymph nodes, colon mucosae, brains or blood. It was present in low concentration in the bronchial lymph nodes of 2 out of 8 animals. This localization of the virus in swine accords with its classification as a pneumotropic virus.

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## EXPERIMENTAL STUDIES ON ENCEPHALITIS

### IV. SPECIFIC INACTIVATION OF VIRUS BY SERA FROM PERSONS EXPOSED TO ENCEPHALITIS, ST. LOUIS TYPE, 1933

By LESLIE T. WEBSTER, M.D., GEORGE L. FITE, M.D., AND ANNA D. CLOW  
WITH A NOTE ON THE EVALUATION OF THE RESULTS OF MOUSE TESTS  
OF SERA

By HUGO MUENCH, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research)*

(Received for publication, July 1, 1935)

Experiments on the probable virus nature of the encephalitis prevalent in St. Louis during the summer of 1933 have been reported by Muckenfuss, Armstrong, and McCordock (1), and by Webster and Fite (2, 3). The former workers obtained a virus by inoculating brain tissue from fatal cases into *Macacus rhesus* monkeys; the latter recovered a similar virus by inoculating the brain tissue into special mice. A further step in establishing this virus as the specific cause of the epidemic was the demonstration by Webster and Fite of the inactivation of the virus by human convalescent sera (4). This finding, confirmed by Wooley and Armstrong (5), and Muckenfuss (6), will now be described in detail.

#### *Technique*

The effect of serum on the virus was studied by means of the familiar protection test. Virus in suitable dilution was mixed with equal volumes of undiluted serum and injected intracerebrally into mice. The survival time of the injected animals was taken as a measure of the protective action of the sera.

Strain 3 virus (2, 3) was used in all experiments since it proved similar to other strains in every respect tested. Brains from one or two mice prostrate 3 to 5 days following an intracerebral injection of mouse brain virus, were triturated with alundum, diluted one part by weight of brain virus to 50 parts by volume of hormone broth, pH 8.0, centrifuged 10 minutes at 1,000 R.P.M., and the supernatant made up further in serial tenfold dilutions with hormone broth. 0.3 cc. of each chosen dilution of the brain virus was mixed thoroughly with 0.3 cc. of serum



in a test tube. The preparations were incubated 2 hours at 37°C., left at room temperature 2 hours, and then each taken up in a 0.25 cc. tuberculin syringe with 0.25 inch 26 gauge needle and injected intracerebrally in 0.03 cc. amounts into four to six mice lightly anesthetized. Precautions for asepsis were observed throughout. The condition and survival time of the injected mice were recorded for 21 days.

The choice of mice proved a significant factor in maintaining a maximum and similar degree of infectivity of the virus and in reducing irregularities in survival time of mice within and between tests. The unselected Rockefeller Institute stock mice (2) maintained the intracerebral infectivity of the virus at varying levels between  $3 \times 10^{-6}$  and  $3 \times 10^{-8}$  gm. of infected mouse brain and gave irregular survival times. Selected Rockefeller Institute resistant (virus-susceptible mice) (2) and selected Swiss mice proved the animals of choice, since they maintained the infectivity of the virus at about  $3 \times 10^{-9}$  gm. of infected mouse brain and proved most uniform in their response to the virus. All mice were free of inter-current infection and came from our own breeding stocks. They were 4 to 6 weeks old and weighed 18 to 22 gm. When, on rare occasions, a doubt arose as to whether an injected animal died of encephalitis, brain sections were taken and tissue passed to another animal to establish the diagnosis. To test the uniformity of the mice, four to six were injected with each virus-serum mixture. To test the infectivity of the virus in each experiment, virus plus non-contact serum mixtures were injected into mice in dilutions of  $10^{-3}$  to  $10^{-7}$  inclusive.

Tests were made as soon as possible after withdrawal of blood, since protective bodies against this virus were found to decrease in quantity with *in vitro* age of serum (3). Protective sera diluted beyond 1 to 10 failed to react; hence tests were made at a final serum concentration of 1 to 2. Uncontrolled variations between experiments were checked by testing a greater part of the unknown sera at least twice, the non-contact control sera three to nine times, the doubtful sera two to four times, and some of the protecting sera two to eight times.

The test as now standardized is made with one non-contact and one known protecting serum as controls, together with five to fifteen test sera. The control sera are prepared to give virus dilutions of  $10^{-5}$  to  $10^{-7}$  inclusive, and the test sera to give virus dilutions of  $10^{-5}$  and  $10^{-6}$ . Each dilution of virus-serum mixture is injected intracerebrally into four Swiss mice.

### *Standardization of Protection Test*

The first few protection tests were devoted primarily to standardization of the technique including the proper manipulation and dilution of the virus, determinations of stability of infectivity of the virus and of protective activity of a given serum, variations in effect of different normal non-contact sera, and variability of results between tests. The protocol of Test 3, for example, published elsewhere (7), besides

demonstrating the protective effect of St. Louis convalescent sera, is an instance in which the titre of the virus was limited to the  $10^{-4}$  dilution by the technique employed. Following these early tests, the titre of virus has remained uniform at the  $10^{-7}$  dilution. Again, it was learned that the protective titre of the human convalescent sera decreased with *in vitro* aging in the same manner as in the case of hyperimmune monkey sera (3).

While this standardization of procedure was being accomplished, it appeared that certain sera protected the injected mice fully against 100 killing doses of virus, but that others protected to a considerably less degree. What, then, was to be the basis of evaluating the protective effect of unknown sera?

The problem, a statistical one, was studied by Dr. H. Muench, of the International Health Division of the Rockefeller Foundation, and the results are given below. The data comprised two to nine titrations on each of thirteen normal non-contact sera, and one to six titrations on each of 267 unknown sera. Of these, 184 were classed later as non-protective and 67 as protective.

#### NOTE ON EVALUATION OF RESULTS OF MOUSE TESTS OF ENCEPHALITIS SERA

In attempting to establish criteria for distinguishing positive (protecting) from negative (non-protecting) sera, the first procedure was to find out what happens to "unprotected" mice that receive virus. For this purpose there was available a set of test results on a series of known normal, non-contact sera which had been used repeatedly as controls for test runs.

It became apparent that conditions in the first six test runs were widely divergent from those in the seventh and following. The latter group was very uniform. For this reason all findings have been based entirely on the analysis of tests after the sixth run. The results in the normal non-contact serum group are summarized in Table I.

Mortality rates are not well determined by one or two deaths even in a group of about 150, so that the rates at  $10^{-4}$  and  $10^{-5}$  cannot be regarded as very definite. The mortality at  $10^{-6}$  is based on only forty-seven mice and therefore it is quite unreliable. The factors most certain, at least for the first two dilutions, are the average time of death after inoculation and its standard deviation.

The tests were done as routine on groups of four or of six mice. Now the standard deviation of the mean time of death in a sample of six mice at  $10^{-4}$  would be  $0.7810/\sqrt{6}$  or 0.3188 days from the mean value of the total. In other words, a mean time of death of 6.01 days would be twice the standard deviation

above the expected and this would occur accidentally in unprotected mice only some twenty-three times in 1000. Likewise, a mean time of death of 6.33 days would exceed the expected by three times the standard deviation; this could be accidental only thirteen times in 10,000.

For  $10^{-5}$  dilutions and for four-mouse groups, the appropriate values are used. In this way it is possible to arrive at the criteria given in Table II. Here the + value corresponds to twice the standard deviation above the average; ++ is three times. Values as large as the latter or larger are almost certainly not due to chance and the corresponding sera cannot be called "negative."

This provides a criterion of what is not a negative serum. The question of what cannot be positive is still to be answered: it is not known whether positive

TABLE I

| Virus dilution | No. of mice | No. dying | Mortality rate | Day of death   |                    |
|----------------|-------------|-----------|----------------|----------------|--------------------|
|                |             |           |                | Average (mean) | Standard deviation |
| $10^{-4}$      | 148         | 147       | 0.9932         | 5.3537         | 0.7810             |
| $10^{-5}$      | 146         | 144       | 0.9863         | 5.9514         | 0.9953             |
| $10^{-6}$      | 47          | 37        | 0.7872         | 6.7027         | 0.8339             |

TABLE II

*Average Survival Time in Days*

| Dilution  | 4 mice |      | 6 mice |      |
|-----------|--------|------|--------|------|
|           | +      | ++   | +      | ++   |
| $10^{-4}$ | 6.13   | 6.53 | 6.01   | 6.33 |
| $10^{-5}$ | 6.95   | 7.44 | 6.76   | 7.17 |

sera behave so differently that the average time of death is necessarily longer than in the case of negatives.

This cannot be answered from a study of known negative sera. It would be difficult to establish the distribution of longevity among "protected" mice from the results of tests of unknown sera, since these are evidently a mixture of positive and negative. A criterion based on mortality rates might be more definite if mortalities in protected and unprotected mice are sufficiently distinct and can be closely evaluated. In addition, such a criterion would be simpler and easier to apply than one based on length of life.

The problem then is to evaluate the two different mortalities at different virus dilutions and to find the point at which there will be the sharpest difference between them. Here there should be the least overlapping of criteria with consequent throwing of results into an "inconclusive" group.

In essence this is a study of binomial distributions. A group of tests, each test comprising the same number of mice among which there is a constant mortality rate would, in the long run, be distributed in a perfectly stable pattern. For example, 1000 groups of six mice each, with a mouse mortality of 0.9, would have the following most probable distribution:

|               |     |
|---------------|-----|
| 6 deaths..... | 532 |
| 5 " .....     | 354 |
| 4 " .....     | 98  |
| 3 " .....     | 15  |
| 2 " .....     | 1   |

while if the mortality were 0.2 we should expect:

|               |     |
|---------------|-----|
| 5 deaths..... | 2   |
| 4 " .....     | 15  |
| 3 " .....     | 82  |
| 2 " .....     | 246 |
| 1 death.....  | 393 |
| 0 deaths..... | 262 |

As a starting point, we may assume that the distribution of test results (by number of mice dying) of any group of unknown sera is made up of a mixture of two such single distributions. One would be the scatter produced by a high mortality in unprotected mice which receive normal serum, which would have a peak at high numbers of deaths. The other, composed of test results in protected mice, would have a peak at a lower mortality. There are three unknown factors to be found: (a) the mortality rate of the unprotected group (= negative sera); (b) the mortality of the other group (protected mice or positive sera); (c) the proportion of each which makes up the entire number of tests; *i.e.*, the number of positive and of negative sera included.

These three factors may be derived from any actual array of test results mathematically in a perfectly straightforward way. Due to sampling variations, the actual distribution of a series of tests would hardly ever be exactly the expected one. For this reason the values we get for the factors are the best values, meaning those which will give the closest approximation to the series we are dealing with since we can hardly expect absolute concurrence.

In Table III is given, as an illustration, an actual distribution; in this case that of 110 tests, using four mice each, at a virus dilution of  $10^{-5}$ . A survivor is a mouse alive on the 21st day after inoculation, when it was dropped from observation. Just below the actual number of tests in each mortality group is the hypothetical number calculated on the basis of the three factors obtained from the actual distribution. It will be seen that the correspondence is very close; the main difference is that there are actually rather more two-death results than might be expected.

If the calculated mortality rates are accurate, it would be expected that any other group of tests at the same dilution would give comparable results. The

third factor (distribution of positives and negatives) would vary from group to group depending on how many positive sera happened to be included in each.

As a matter of fact, the unprotected mortality is very constant. This is illustrated in Table IV which summarizes the values obtained by analyzing different sets of data. Four-mouse and six-mouse groups must be separated for study on a binomial basis. The two different strains of mice were also kept separate in

TABLE III  
*Results of Four-Mouse Tests at  $10^{-5}$  (Swiss Mice)*

| No. of mice dying.....     | 4    | 3    | 2   | 1    | 0    | Total |
|----------------------------|------|------|-----|------|------|-------|
| No. of tests (actual)..... | 67.0 | 10.0 | 7.0 | 9.0  | 17.0 | 110.0 |
| " " " (calculated).....    | 66.5 | 12.2 | 3.7 | 11.1 | 16.5 | 110.0 |

Calculated rates on basis of: 0.9571 mortality in 79 negative tests.

0.1455 " " 31 positive "

TABLE IV  
*Mortality Rates Derived from Test Results on "Unknown" Sera*

| Dilution           | Mouse strain | No. of tests | Calculated mortality rates |               | "Unprotected" rates from normal sera (Table I) |
|--------------------|--------------|--------------|----------------------------|---------------|--|
|                    |              |              | "Protected"                | "Unprotected" |  |
| $10^{-4}$ : 6 mice | VS           | 33           | 0.6504                     | 1.0           | 0.9932   |
|                    | Swiss        | 95           | 0.4485                     | 0.9943        |  |
|                    | VS           | 27           | 0.1382                     | 0.9841        |  |
|                    | Swiss        | 99           | 0.6954                     | 1.0           |  |
| $10^{-5}$ : 6 mice | VS           | 43           | 0.1712                     | 0.9717        | 0.9863   |
|                    | Swiss        | 92           | 0.2800                     | 0.9760        |  |
|                    | VS           | 27           | 0.0639                     | 0.9463        |  |
|                    | Swiss        | 110          | 0.1455                     | 0.9571        |  |
| $10^{-6}$ : 4 mice | VS           | 27           | 0.0739                     | 0.9186        | 0.7872   |
|                    | Swiss        | 67           | 0.1076                     | 0.9153        |  |

case there should be a difference in their reactions. Known normal sera were excluded.

Not only are the unprotected mortalities homogeneous within a given dilution; they agree quite well with the mortality rates in known normal sera as given in Table I. The average values of 0.9640 at virus dilutions of  $10^{-5}$  and 0.9160 at  $10^{-6}$  are probably very close to the true mortalities. This permits the statement that, in negative sera, the expected occurrence of surviving mice would be as given in Table V. Unprotected mortality appears to have a fixed value and the

occurrence of five or six survivors in a test group of six mice, for instance, would throw a serum out of all reasonable probability of being negative.

The answer to the search for a protected mortality is nothing like as clear. It appears that there is no such entity, but rather a band of mortalities covering a considerable range which may reflect variations in protective power in different sera. Such spreading is indicated by the wide fluctuations between the calculated values for different groups in the same dilution (Table IV). The number of positive tests in each group is comparatively small and the variations show the effects of sampling from a widely spreading field. Confirmation of this conception of protected mortality as a band instead of a point is found in the quite regular excess of actual over computed results around the 50 per cent mortality point, which is shown in Table III.

But the computed protected mortality must be a "centering constant" of some sort. That is, it must be somewhere around the middle of the band of mortalities, some values being higher and some lower. In that case the lower the computed

TABLE V  
*Expected Survivors with Negative Sera*

| Dilution                  | 2 or more | 3 or more | 4 or more          |
|---------------------------|-----------|-----------|--------------------|
| 10 <sup>-4</sup> : 6 mice | 176       | 8         | — per 10,000 tests |
| 4 "                       | 74        | 2         | — " 10,000 "       |
| 10 <sup>-5</sup> : 6 "    | 842       | 97        | 6 " 10,000 "       |
| 4 "                       | 378       | 23        | 1 " 10,000 "       |

value, the less spread is to be expected. If the computed mortality is 0.5, the scatter of actual values might conceivably be quite even from 0 to 1.0. But if its value is 0.1, then it cannot be an average of a series which runs heavily to high values.

From this viewpoint it is possible to discuss results at different virus dilutions and their bearing on setting up criteria.

Dilution 10<sup>-4</sup>. Negative sera produce scarcely any survivors, but the mortality among protected mice is so high that many positive sera, especially if weakly protective, will show few or no survivors and so cannot be differentiated.

Dilution 10<sup>-5</sup>. The mortality in negative sera is still high, so that a test running to three or more survivors is quite certainly positive (Table V). Protected mortalities are evidently at a lower level than at 10<sup>-4</sup>, but it might be expected that a weakly protective serum would show a sufficient number of deaths to throw the results into the possibly negative category.

Dilution 10<sup>-6</sup>. Although lower, unprotected mortality is still sufficiently high to make the occurrence of three or more survivors indicative of a positive

serum. Protected mortality now centers about a value around 0.1 or less and in all probability seldom is high enough to confuse protective with non-protective sera.

Results at virus dilutions of  $10^{-6}$  are thus undoubtedly the most sensitive and delicate. Dilutions were not carried farther than this: at  $10^{-7}$  protected mortality would be lower but so would unprotected, and there might be less certainty in differentiating between the two than at  $10^{-6}$ . It is impractical to embody dilutions of  $10^{-4}$  in the criterion as the two mortality rates are so nearly alike.

As finally set up, the criterion of positive protection on the basis of mortality is as follows:

TABLE VI  
*Criterion for Test Results*

| No. of survivors*..... | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|------------------------|---|---|---|---|---|---|---|
| $10^{-5}$ : 6 mice     | — | — | ± | + | + | + | + |
| 4 "                    | — | — | + | + | + |   |   |
| $10^{-6}$ : 6 "        | — | — | — | + | + | + | + |
| 4 "                    | — | — | ± | + | + |   |   |

\* At 21 days.

A number of sera, by this criterion, are positive at  $10^{-6}$  but negative at  $10^{-5}$ . This would be expected in the case of weakly protective sera according to the interpretation of the analysis. The reverse result should be found very seldom and it has not, in fact, occurred so far. Confirmation of the conception of such positive-negative results as weak positives is seen in the fact that the same sera are almost invariably outside the range of possible negatives on the basis of average length of life (Table II).

A ± result (Table VI) must be regarded as inconclusive unless the result at another dilution is clearly positive.

The final criterion for use in practise may be established as follows:

- + at  $10^{-5}$  and + at  $10^{-6}$ : ++ (strongly positive)
- at  $10^{-5}$  and + at  $10^{-6}$
- ± at  $10^{-5}$  and + at  $10^{-6}$  } : + (weakly positive)
- at  $10^{-5}$  and ± at  $10^{-6}$ : ± (inconclusive, probably negative)
- at  $10^{-5}$  and — at  $10^{-6}$ : — (negative, no protection)

where the +, ±, and — values are determined from the number of survivors as given in Table VI.

#### *Results with Sera from Persons with No History of Exposure to St. Louis Encephalitis*

Sera were collected from September, 1933, to date from healthy non-contacts and from cases of encephalitis and poliomyelitis mostly in

hospitals in eastern and north central states.<sup>1</sup> The cases chosen were believed to have received careful clinical study, a matter of prime importance in determining the specificity of the reaction between serum and virus. The sera from the healthy non-contacts described below, when mixed with virus in dilutions of  $10^{-4}$  to  $10^{-6}$  and injected into mice, gave mortality rates of 99 per cent, 98 per cent, and 78 per cent, respectively, and average survival times in days of 5.3, 5.9, and 6.7 (Table I). The other sera, under similar conditions, gave mortality rates of 99 per cent, 96 per cent, and 91 per cent (Table IV), indicating that practically all mice given these sera plus virus in dilutions as low as  $10^{-6}$  die in 5 to 7 days.

TABLE VII  
*Criteria for Protective Serum*

| Dilution of virus | Average survival time |              | No. of survivors |              |
|-------------------|-----------------------|--------------|------------------|--------------|
|                   | 4 mice tests          | 6 mice tests | 4 mice tests     | 6 mice tests |
|                   | <i>days</i>           | <i>days</i>  |                  |              |
| $10^{-4}$         | 6.53                  | 6.33         | —                | —            |
| $10^{-5}$         | 7.44                  | 7.17         | 2                | 3            |
| $10^{-6}$         | —                     | —            | 3                | 3            |

*Healthy Non-Contacts.*—Sera from thirteen adults working in medical institutions in or near New York have been tested one to eight times with negative results (Table VII). Protocols of repeated tests with three of these sera are given in Table VIII, showing the uniformity of results subsequent to Test 14. In

<sup>1</sup> The authors are grateful for the generous cooperation of physicians and hospital authorities in calling our attention to cases of encephalitis, collecting and sending sera, and supplying clinical data. Mentioning all these collaborators by name is regarded as impractical. Most of the sera from the cases of chronic encephalitis with Parkinson sequelae were sent by Dr. David Marine, Montefiore Hospital, New York; Dr. C. H. Andrewes, National Institute for Medical Research, London; Dr. E. A. Carmichael, National Hospital Queen Square, London; Drs. Josephine B. Neal and Frederick Tilney, Neurological Institute, New York; and Dr. M. W. Raynor, Bloomingdale Hospital, White Plains, N. Y. Professors R. Inada and K. Kakinuma sent sera from twelve cases of Japanese B encephalitis, and Professor I. Takaki sent sera from three similar cases plus samples of hyperimmune sera and virus A and B in glycerine. Dr. G. F. Kempf forwarded sera from cases of meningoencephalopathy in Indianapolis.



TABLE VIII

*Repeated Tests with Three Sera from Healthy Non-Contacts*

| Serum | Test | Date     | Virus-serum dilution |                     |                     |                     |                  |
|-------|------|----------|----------------------|---------------------|---------------------|---------------------|------------------|
|       |      |          | 10 <sup>-3</sup>     | 10 <sup>-4</sup>    | 10 <sup>-5</sup>    | 10 <sup>-6</sup>    | 10 <sup>-7</sup> |
| C     |      | 1933     |                      |                     |                     |                     |                  |
|       | 13   | Nov. 23  | 4, 5, 6, 7           | 5, 5, 5, 6          | 5, 6, 6, S          |                     |                  |
|       | 14   | " 29     | 4, 4, 4, 6           | 4, 5, 6, 7          | 6, 6, 9, S          |                     |                  |
|       | 16   | Dec. 20  |                      | 4, 5, 5, 5,<br>6, 6 | 5, 5, 5, 6,<br>*, * |                     |                  |
|       |      | 1934     |                      |                     |                     |                     |                  |
|       | 28   | Apr. 25  |                      | 5, 5, 5, 6,<br>6, 6 | 6, 6, 6, 6,<br>7, 7 |                     |                  |
|       | 43   | Dec. 4   |                      | 6, 6, 6, 6          | 6, 6, 6, 7          | 7, 7, 7, 8          | 8, S, S, S       |
|       |      | 1935     |                      |                     |                     |                     |                  |
|       | 45   | Jan. 18  |                      | 5, 5, 5, 6          | 6, 6, 6, 7          | 6, 6, 7, 8          | 6, 6, 11, 12     |
|       | 47   | Mar. 7   |                      | 5, 5, 6, 6          | 6, 6, 6, 6          | 5, 7, 7, 7          | 8, 8, S, S       |
|       | 48   | " 12     |                      | 5, 5, 5, 5          | 5, 5, 5, 6,<br>6, 7 | 6, 6, 7, 7,<br>7, 9 | 7, 8, 8, 9       |
|       | 49   | May 8    |                      | 6, 6, 6, 6          | 5, 6, 6, 7          | 6, 6, 7, 7          | 7, 8, S, S       |
|       | 50   | " 24     |                      | 5, 5, 6, 6          | 6, 6, 7, 7          | 7, 7, 9, 9          | 8, 10, S, S      |
|       | 51   | June 5   |                      | 5, 6, 6, 6          | 5, 6, 7, 7          | 5, 6, 6, 7          | 6, 6, 7, 7       |
|       | 52   | " 10     |                      | *, 5, 5, 6          | 5, 5, 6, 6          | 5, 6, 6, 7          | 6, 7, 8, 8       |
| J     |      | 1934     |                      |                     |                     |                     |                  |
|       | 28   | Apr. 25  |                      | 5, 6, 6, 6,<br>6, 6 | 6, 6, 6, 8,<br>8, 8 |                     |                  |
|       | 36   | Oct. 10  |                      | 5, 5, 5, 6,<br>6, * | 5, 5, 5, 6,<br>6, 6 |                     |                  |
|       | 39   | " 25     |                      | 4, 5, 5, 5          | 4, 5, 5, 7          | 6, 6, 7, 7          |                  |
|       |      | " 30     |                      | 4, 6, 6, 8          | 6, 6, 6, 6          |                     |                  |
| H     |      | 1935     |                      |                     |                     |                     |                  |
|       | 46   | Jan. 31  |                      | 5, 6, 6, 6          | 5, 6, 6, 6          | 6, 7, 8, 8          | 6, 8, 9, S       |
|       |      | 1933     |                      |                     |                     |                     |                  |
|       | 12   | Nov. 16  | 4, 5, 5, 6           | 5, 5, 6, 6          | 5, 6, 6, 6          | 5, 5, 7, 9          |                  |
|       |      | 1934     |                      |                     |                     |                     |                  |
|       | 28   | Apr. 25  |                      | 6, 6, 6, 6,<br>6, 7 | 6, 6, 6, 6,<br>7, 7 |                     |                  |
|       | 33   | Sept. 25 |                      | 5, 5, 5, 5,<br>5, 5 | 5, 5, 5, 6,<br>6, 9 |                     |                  |
|       | 41   | Nov. 12  |                      | 5, 5, 5, 5          | 5, 5, 7, 7          | 7, 7, 7, 7          | 7, 7, 7, 7       |

S = mouse remained well 21 days.

Blank spaces indicate dilution not tested.

\* = mouse died from trauma.

contrast to these findings reported in 1933 (4), Wooley and Armstrong state (1934) (5) that eleven of 113 sera (9.7 per cent) from individuals with no special contact with cases gave strong or moderate protection. This discrepancy will be discussed later.

*Chronic Lethargic Encephalitis with Parkinson Sequelae.*—Webster and Fite reported in 1933 (4) that sera from sixteen cases of lethargic encephalitis with Parkinson sequelae failed to protect mice against the virus. Similar results were reported by Levaditi, Schoen, and Levaditi (1934) (8) on sera from four cases. Wooley and Armstrong, however, state (1934) (5) that sera of four of twenty-nine (13.7 per cent) tested cases showed strong protection.

We have now studied a total of twenty-seven sera from typical cases. The results are negative. Nine cases gave a history of onset following an attack of influenza in 1918 to 1920. All have had Parkinson sequelae for 1 to 15 years.

*Typical, Acute Encephalitis (Economo).*—The negative effect of sera from the twenty-seven chronic cases of outspoken encephalitis of the Economo type raised the question of whether sera from the same disease in the more acute stages would show protective substances against the St. Louis virus. Realizing that a clinical diagnosis of this disease in the acute stages is often difficult, an effort was made to obtain sera from both typical and atypical cases.

Eight typical acute cases were tested and found to be negative. All gave histories of lethargy, ptosis or diplopia, tremors, and mononuclear pleocytosis of the spinal fluid. Two had beginning mask facies. Blood was drawn for testing 2, 2, 4, 6, 10, 12, 16, and 26 weeks after onset.

*Atypical Primary Encephalitis.*—Sixty-nine cases of atypical primary encephalitis were also tested with negative results. Symptoms and signs varied widely but were of such a nature in each case as to warrant a clinical impression of "encephalitis." Three cases occurred in 1932, twenty-eight in the autumn of 1933, thirty in 1934, and eight in 1935. Sera for testing were obtained 2 to 52 weeks after onset of illness. Twenty-four were from New York, two from New Jersey, six from Connecticut, two from Massachusetts, one from Pennsylvania, five from Maryland, four from Virginia, one each from Florida and Alabama, five from Illinois, four from Missouri, one from Indiana, eleven from Ohio, and two from California.

*Japanese B Encephalitis.*—We found (1934) (9) that sera from fifteen cases of Japanese B encephalitis did not protect against the St. Louis virus. These results were surprising in view of the reported similarity in epidemiological and clinical features of the Japanese and St. Louis diseases (10).

Sera were received from three persons supposed to have had the disease in August, 1924, aged 50, 51, and 60 years, and from nine persons convalescent from the August, 1933, epidemic, aged 17, 17, 20, 26, 33, 46, 53, 62, and 65 years. In these cases fever subsided 6 to 12 days after onset of symptoms. Sera were likewise received from three additional persons convalescent from the 1933 epidemic. Blood specimens were drawn from the 1924 cases about 10 years after onset and from the 1934 case about 4 months after onset. Specimens were tested after

about 6 weeks' aging *in vitro*. Each serum was tested twice with negative results. Kodama has recently confirmed these findings using Strain 3 virus and serum from convalescents in Japan (13). Further negative tests on sera from animals immunized with Takaki's B and A viruses (11), and futile attempts to establish the B virus from glycerinized material are reported below.

*Postinfectious Encephalitis*.—Sera from ten cases of postinfectious encephalitis were tested and found negative. Three cases were encephalitis following herpes zoster; two additional cases of herpes zoster without encephalitis were found negative. Four cases were post-measles encephalitis, two post-pertussis encephalitis, and one encephalitis complicating varicella.

*Meningoencephalopathy, Indianapolis*.—Sera from two convalescents from meningoencephalopathy at Indianapolis described by Kempf, Gilman, and Zervas (12) did not protect against the virus (4).

*Australian "X" Disease*.—Serum from a case reported to have had "X" disease was found negative.

*Poliomyelitis, Los Angeles, 1934*.—Sera from eleven cases of poliomyelitis in Los Angeles, 1934, were obtained 4 weeks and 8 months after onset of symptoms. All were negative.

In summary, none of the 156 tested sera from persons believed to have had no exposure to St. Louis encephalitis has shown protective antibodies against the virus. Wooley and Armstrong's series of similar cases (*a*) lethargic encephalitis, (*b*) unclassified encephalitis, acute meningoencephalitis, epidemic meningoencephalopathy, poliomyelitis, traumatic encephalitis, postinfectious encephalitis, Jacksonian epilepsy, (*c*) other diseases not neural, and (*d*) normal controls with no special contact, gave positives at the rates of 13.7 per cent of 29, 11.4 per cent of 34, 13.1 per cent of 99, 9.7 per cent of 113, respectively, or 11 per cent of the total 275.

#### *Results with Sera from Animals Immunized with Known Viruses*

The protection test was used not only to determine the specificity of the reaction between serum and virus but to discover a serological relationship between the St. Louis and other known viruses. Sera from immunized animals known to protect against the homologous virus<sup>2</sup> were tested against the St. Louis virus. The results were nega-

<sup>2</sup> The herpes sera were supplied by Dr. Margaret Holden, and the Japanese encephalitis A and B sera by Professor I. Takaki. Dr. C. TenBroeck sent us the equine encephalomyelitis sera and carried out the protection tests with the encephalomyelitis virus. We are indebted to Dr. P. J. du Toit, Pretoria, South

tive, indicating no serological relation between this and the following viruses: herpes, Japanese B and A, poliomyelitis, equine encephalomyelitis, vesicular stomatitis, louping ill, blue tongue, and fox encephalitis.<sup>2</sup>

*Herpes*.—Sera from six rabbits immunized with the E. L. 1 Perdrau strain were tested with negative results.

*Japanese Encephalitis A and B*.—One specimen each of anti-A and anti-B goat serum was received for testing. The anti-A serum run on two occasions did not protect against the St. Louis virus. The anti-B serum contained preservative and gave some protection against St. Louis encephalitis, louping ill, and yellow fever viruses. When injected without virus into mice, it induced transient convulsions.

Besides sera, specimens of virus A and virus B were received. One lot of B virus in rabbit brain preserved in glycerin was received Feb. 8, 1934, and injected into six white-face mice and three 800 gm. rabbits intracerebrally, intracutaneously, and intracorneally. The animals remained well. Later, the material was injected into three 800 gm. rabbits intracoronitidly, into three subdurally, into three intracerebrally, into two intratesticularly, and into two intravenously. All remained well. A second lot of B virus and a specimen of A virus were received May 11, 1934, and each injected subdurally, intracerebrally, intracoronitidly, and intratesticularly into a total of twenty-five 800 gm. rabbits. No virus could be demonstrated.

*Poliomyelitis*.—Sera from two *Macacus rhesus* monkeys immunized by repeated injections of the M. V. virus did not protect against the St. Louis virus.

*Equine Encephalomyelitis*.—Sera from a rabbit immunized with the western strain and from a rabbit and horse immunized with the eastern strain were reported negative by Cox and Fite (14). Later tests have now been made with sera from a guinea pig immunized to the western strain and from a horse immunized to the eastern strain. These likewise showed no protective effect against the St. Louis virus. Here it should also be stated that sera from monkeys immunized with the St. Louis virus failed to protect guinea pigs against the two strains of encephalomyelitis virus (14).

*Vesicular Stomatitis*.—Serum from a rabbit immunized with the New Jersey strain and one from a rabbit immunized with the Indiana strain were reported negative by Cox and Fite (14). Serum from a monkey immunized with the St. Louis virus failed to protect guinea pigs against the vesicular stomatitis strains (14).

*Louping Ill*.—Serum from a horse convalescent from an experimental infection of louping ill showed no protective activity against the virus. In addition, sera

Africa, for the louping ill and blue tongue sera, and to Dr. R. G. Green for the fox encephalitis sera.

from four persons showing specific protective substances against the louping ill virus (15) failed to protect against the St. Louis virus. Finally, anti-St. Louis monkey serum did not protect mice against the louping ill virus.

*Blue Tongue*.—Serum from sheep immunized with the routine passage virus said to be antigenically similar to all other known strains did not protect against the St. Louis virus.

*Fox Encephalitis*.—Sera from a normal fox and a fox immunized with fox encephalitis virus were negative.

*Results with Sera from Persons with History of Exposure to the St. Louis Type of Encephalitis<sup>3</sup>*

*St. Louis Cases, 1933*.—Sera from thirty-six cases on the encephalitis wards of the St. Louis City Isolation Hospital during the epidemic in August and September, 1933, were obtained for study. Eight cases diagnosed as not encephalitis of the prevailing type showed no protective properties in their sera. The remaining twenty-eight cases presented the clinical picture characteristic of the majority of cases of the epidemic (10); namely, high incidence among adults (17, 18, 22, 23, 28, 30, 31, 33, 36, 37, 38, 40, 49, 49, 50, 53, 55, 55, 60, 62, 64, 68, 75, and 75 years), systemic reactions including fever, headache, and vomiting, stiff neck and tongue tremors, and a lymphoid cell pleocytosis of spinal fluid. 82.5 per cent of the twenty-eight cases showed protective properties against the virus (Table IX). The negative cases were aged 23, 36, 40, 49, and 75 years. If the cases are grouped according to whether the first bleeding was made before or after the 14th day from onset, the seventeen sera drawn on the 14th day or later all protect, while of the eleven drawn less than 14 days from onset, only six, 54.5 per cent protect.

The negative effect of the "early" sera is not understood. The

<sup>3</sup> Sera from St. Louis cases of encephalitis, 1933, were obtained through the courtesy of Drs. R. S. Muckenfuss, J. Eschenbrenner, Jr., and S. Weisman. Dr. P. F. Stookey sent us sera from cases of encephalitis of the St. Louis and other types occurring in Kansas City, and brain tissue from fatal cases. Dr. H. D. McIntyre forwarded sera from sixteen cases of encephalitis in Cincinnati, 1933 and 1934. Sera from five cases of encephalitis in Paris, 1932, were sent by Dr. W. E. Conklin; later, sera from fifty cases of encephalitis of the St. Louis type in Paris, Danville, and Canton, Illinois, were sent by Dr. W. H. Tucker through the courtesy of Dr. F. J. Jirka. The Indiana State Board of Health kindly supplied sera from five cases of the disease.

view was taken in an earlier report that insufficient time had elapsed for the development of antibodies (4), but this is now untenable since second bleedings from these same cases 4 months after onset likewise gave negative results. Such explanations as inability of certain persons to elaborate demonstrable antibodies or incorrect diagnoses

TABLE IX  
*Sera Tested against Encephalitis Virus (St. Louis Type)*

| Diagnosis                                | No. tested | No. positive | Per cent positive |
|--|------------|--------------|-------------------|
| Healthy non-contacts.....                | 13         | 0            | 0                 |
| Chronic encephalitis Economo.....        | 27         | 0            | 0                 |
| Acute " ".....                           | 8          | 0            | 0                 |
| Atypical, suspected encephalitis.....    | 69         | 0            | 0                 |
| Japanese B encephalitis.....             | 15         | 0            | 0                 |
| Postinfectious encephalitis.....         | 10         | 0            | 0                 |
| Meningoencephalopathy, Indianapolis..... | 2          | 0            | 0                 |
| Australian "X" disease.....              | 1          | 0            | 0                 |
| Poliomyelitis, Los Angeles, 1934.....    | 11         | 0            | 0                 |
| Immunized animals.....                   | 21         | 0            | 0                 |
| St. Louis not encephalitis, 1933.....    | 8          | 0            | 0                 |
| " " encephalitis, 1933.....              | 28         | 23           | 82.5              |
| Kansas City encephalitis, 1933.....      | 4          | 3            | 75.0              |
| Cincinnati " 1933.....                   | 1          | 1            | 100.0             |
| New York " 1933.....                     | 2          | 2            | 100.0             |
| Paris, Ill. " 1932.....                  | 12         | 11           | 91.6              |
| " " " 1934.....                          | 9          | 6            | 66.6              |
| Danville, Ill. " 1934.....               | 21         | 14           | 66.6              |
| Canton, Ill. " 1934.....                 | 6          | 3            | 50.0              |
| Indiana " 1934.....                      | 5          | 5            | 100.0             |
| Cincinnati " 1934.....                   | 4          | 2            | 50.0              |
| California " 1934.....                   | 3          | 1            | 33.3              |

appear most probable, even though the negative reactors were concentrated in the less than 14 day group.

These results are not in complete agreement with those of Muckenfuss (6). Fifteen of the thirty-four sera were tested by both workers with agreement in the case of eleven and in the case of four, positive results by Muckenfuss and negative by ourselves, a disagreement of 26.6 per cent. We tested two of the disputed sera three times on two specimens; the other two, once only.

*Kansas City Cases, 1933.*—Encephalitis, so conspicuous in St. Louis during August and September, 1933, was present at the same time in Kansas City. Here, however, fewer cases were recorded and the clinical pictures were more varied. From one of two tested fatal cases, a virus was recovered similar to that obtained from fatal cases in St. Louis (2). At the same time sera from four outspoken cases were tested against the St. Louis virus and three found positive (4).

*Ohio Case, 1933.*—A white male, aged 41, contracted encephalitis August 30, in Cincinnati, of a type similar to the St. Louis disease. His serum, collected and tested 1 year later, October, 1934, neutralized the virus.

*New York Cases, 1933.*—Recognition of cases of the St. Louis type of encephalitis naturally became more difficult in places remote from the epidemic. Tests were made, therefore, on sera from various forms of atypical encephalitis, sixteen in all, from September to the end of December, 1933. Of these, only two were considered clinically as possible cases of the St. Louis disease.

No. 47. M. S., a white male, aged 27, was admitted to St. Luke's Hospital, Dr. Frissell's service, on Sept. 9, 1933, with a 3 day history of nausea, vomiting, fever, malaise, generalized throbbing headache, and no history of having been to St. Louis or having come in contact with anyone from St. Louis. On admission his temperature was 101°F. There was a slight leucocytosis, 10,900, 80 per cent polymorphonuclears. The Wassermann reaction was negative. The other findings were chiefly neurological and consisted of absent abdominal reflexes, hyperactive tendon reflexes, medium coarse nystagmus, soreness but no stiffness of neck, a suggestive Kernig, and weakness of flexion of right arm. Spinal fluid, pressure 240; cells 144 (lymphoid cells 96 per cent), globulin +, protein 82, sugar 58. Within a week's time all symptoms and signs had returned to normal, save for hyperactivity of tendon reflexes and persistence of nystagmus. The cell count of the spinal fluid had dropped to 50 in 3 weeks, and to 15 in 6 weeks. Dr. Frissell called this case to our attention as possible St. Louis encephalitis and tests on sera drawn 2 and 6 months after onset, ten in all, were strongly positive (4), (Table X).

No. 63. B., a white female, aged 39. After 3 weeks' vacation in Kentucky, returned to New York with severe headache. She was seen by Dr. H. T. Chickering on Sept. 12, 1933. Her temperature was 103°F. She complained of severe headache, different from any other, accompanied by chills. This condition cleared up rapidly and she was discharged as well on Sept. 25. Dr. Chickering mentioned this case as possible mild encephalitis of the St. Louis type. Two tests on serum drawn 11 weeks after onset were positive (4), (Table X).

*Paris, Illinois, Cases, 1932.*—In searching for a possible relationship between the 1933 St. Louis and previous outbreaks of encephalitis, mention has been made of protection tests run on sera from cases of Economo encephalitis with onset following influenza in 1918, and meningoencephalopathy in Indianapolis. The results were negative. The case of the outbreak of encephalitis in Paris, Illinois, in 1932, however, was different. Encephalitis appeared in Paris, Illinois, a

TABLE X

*Positive Protection of Sera from New York, 1933, Cases against the St. Louis Virus*

| Sera No. | Test No | Date of test   | Virus-serum dilution |                  |                  |                  | Diagnosis                        |
|----------|---------|----------------|----------------------|------------------|------------------|------------------|----------------------------------|
|          |         |                | 10 <sup>-1</sup>     | 10 <sup>-2</sup> | 10 <sup>-3</sup> | 10 <sup>-4</sup> |                                  |
| 47       | 8       | 1933<br>Nov. 2 | 6, 7, S, S           | 6, S, S, S       | S, S, S, S       | S, S, S, S       | Acute encephalitis (St. Louis?)  |
| 46       | 8       | " 2            | 4, 4, 4, 6           | 5, 5, 5, 5       | 5, 5, 6, 6       | 6, 6, 7, 9       | Chronic encephalitis (Parkinson) |
| 47       | 12      | " 16           | 5, 6, 7, 7           | 7, 7, 9, 10      | S, S, S, S       | S, S, S, S       | Acute encephalitis (St. Louis?)  |
| H        |         |                | 4, 5, 5, 6           | 5, 5, 6, 6       | 5, 6, 6, 6       | 5, 5, 7, 9       | Normal non-contact               |
| 63       | 13      | Nov. 23        | 5, 6, 6, 7           | 6, 7, 7, 7       | S, S, S, S       | S, S, S, S       | Acute encephalitis (St. Louis?)  |
| 58       |         |                | 5, 5, 5, 7           | 5, 5, 6, 8       | 5, 5, 6, 6       | 6, 7, 9, 10      | Chronic encephalitis (Parkinson) |

S = mice remained well 21 days.

community of about 9000 persons, in July and August, 1932 (16, 10). Twenty-seven persons were affected, aged 33 to 80, with only three under 50. The mortality was close to 50 per cent, but convalescents were relatively free of sequelae. The outstanding symptoms recorded were headache, fever, nausea and vomiting, diplopia, and delirium or stupor. Facies were set, neck rigid, and tongue tremulous. Sera from five convalescents drawn 15 months after onset were tested against the St. Louis virus. Four of the five gave definite protection (4), (Table XI). Later, February, 1935, 2½ years after the outbreak,



TABLE XI  
Protection Tests on Sera from Cases in Paris, Illinois, 1932 and 1934

| Serum         | Test | Virus-serum dilution |                  |                  |                  | Result |
|---------------|------|----------------------|------------------|------------------|------------------|--------|
|               |      | 10 <sup>-4</sup>     | 10 <sup>-5</sup> | 10 <sup>-6</sup> | 10 <sup>-7</sup> |        |
| C-non-contact | 47   |                      |                  |                  |                  |        |
| Monkey immune |      |                      |                  |                  |                  |        |
| 246 Paris '31 |      | 5, 5, 6, 6           | 6, 6, 6, 6       | 5, 7, 7, 7       | 8, 8, S, S       | 0      |
| 242 " '32     |      | 6, 6, 8, 8           | 6, 7, 8, S       | S, S, S, S       | S, S, S, S       | +      |
| 248 " '32     |      | 4, 5, 5, 5           | 6, 6, 7, 9       | 6, 7, 7, 9       |                  | 0      |
| 247 " '32     |      | 9, 10, S, S          | S, S, S, S       | S, S, S, S       |                  | ++     |
| 241 " '32     |      | 9, 9, 9, S           | S, S, S, S       | S, S, S, S       |                  | ++     |
| 239 " '34     |      | 7, 9, 9, 9           | S, S, S, S       | S, S, S, S       |                  | ++     |
| 244 " '34     |      | 5, 5, 8, S           | 6, 7, 9, 9       | S, S, S, S       |                  | +      |
| 240 " '34     |      | 6, 6, 6, 7           | 6, 8, 9, S       | S, S, S, S       |                  | +      |
| 243 " '34     |      | 6, 7, 7, 8           | 6, 6, 7, 7       | 10, 5, S, S      |                  | +      |
| 245 " '34     | 48   | 6, 6, 6, 7           | 5, 7, 9, 9       | 6, 7, 7, 8       |                  | 0      |
| C-non-contact |      |                      |                  |                  |                  |        |
| 251 Paris '32 |      | 4, 5, 5, 6           | 6, 6, 6, 6       | 6, 6, 6, 6       |                  | 0      |
| 250 " '32     |      | 5, 5, 5, 5           | 5, 5, 5, 6, 6, 7 | 6, 6, 7, 7, 9    | 7, 8, 8, 9       | 0      |
| 257 " '32     |      | 6, 8, 8, 8           | S, S, S, S, S, S | S, S, S, S, S, S |                  | ++     |
| 252 " '32     |      | 5, 7, 8, 8           | 6, 9, S, S, S, S | S, S, S, S, S, S |                  | ++     |
| 253 " '32     |      | 7, 8, 8, 9           | 9, 9, 9, S, S, S | S, S, S, S, S, S |                  | +      |
| 256 " '32     |      | 5, 5, 8, 8           | 5, 6, 7, 7, 8, 8 | 6, S, S, S, S, S |                  | +      |
| 258 " '34     |      | 5, 5, 6, 7           | 6, 6, 6, 7, 8, 8 | 9, S, S, S, S, S |                  | +      |
| 249 " '34     |      | 5, 5, 5, 5           | 5, 6, 6, 7, 8, 8 | 8, 8, 8, 8, 8, S |                  | 0      |
| 255 " '34     |      | 7, 8, 8, 8           | 9, S, S, S, S, S | S, S, S, S, S, S |                  | ++     |
| 254 " '34     |      | 5, 7, 8, 9           | 9, S, S, S, S, S | 8, S, S, S, S, S |                  | ++     |
|               |      | 6, 7, 7, 8           | 7, 8, 8, 8, 8    | 7, S, S, S, S, S |                  | ++     |
|               |      | 6, 6, 8, 8           | 6, 7, 7, 9, 9, 9 | 7, 8, 8, S, S, S |                  | +      |

S = mice remained well 21 days.

Blank spaces indicate dilution not tested.

additional sera were obtained from ten of the cases, including three of the five previously tested. Nine of the ten or eleven of the total twelve (91.6 per cent) were positive (Table XI). Wooley and Armstrong found ten of eleven (90.9 per cent) positive (5).

*Illinois, Ohio, and Indiana Cases, 1934.*—The reappearance of encephalitis in the north central states during the summer of 1934 afforded another opportunity to study the specificity of the serum reaction. Cases clinically resembling the St. Louis type were occurring in localized outbreaks in Illinois and sporadically in Ohio and Indiana.

*Paris, Illinois.*—Nine convalescents, aged 10, 15, 33, 35, 38, 44, 68, 70, and 76 years, were bled 5 to 6 months after onset of symptoms and their sera tested against the St. Louis virus. Six of the nine specimens, 66.6 per cent, gave definite protection. The negative cases were aged 10, 38, and 70 years. If the 10 year old case is omitted, the percentage of positives becomes 75.

*Danville, Illinois.*—Twenty-one convalescents, aged 9, 10, 15, 16, 19, 20, 22, 26, 26, 27, 30, 30, 31, 31, 32, 33, 36, 38, 40, 59, and 60 years respectively, were tested 2 months after onset of symptoms. Fourteen of the twenty-one, 66.6 per cent, showed protective properties in their sera. The negative cases were aged 9, 10, 15, 16, 22, 38, and 40 years respectively. If the four cases aged 16 years or less are omitted in the count, the ratio of positive reactors is increased to fourteen of seventeen, or 82.5 per cent.

*Canton, Illinois.*—Sera were obtained from six convalescents aged 7, 12, 51, 58, 75, and 80 years. Those aged 58, 75, and 80 years protected (50 per cent). If the 7 and 12 year cases are omitted from the count, the ratio of positives becomes three of four (75 per cent).

In summary, of thirty-six Illinois 1934 cases tested, seven were aged 16 years or less and did not protect; of the twenty-nine remaining however, twenty-three (79.3 per cent) were positive.

*Indiana Cases.*—Sera from five cases drawn at least 4 weeks after onset were tested and found to protect against the virus.

*Ohio Cases.*—Sera from two of four typical cases drawn 1 to 3 months after onset neutralized the virus.

*California Cases, 1934.*—Sera from one of three cases protected against the St. Louis virus.

#### DISCUSSION

The specificity of the encephalitis protection test is indicated by the present work and by reports of Wooley and Armstrong and Muck-

enfuss. The latter workers, however, record a 10 to 30 per cent incidence respectively of positive reactors among groups of persons with no special exposure to the St. Louis disease. This discrepancy is due either to differences in batches of sera tested, or more probably to differences in technique and criteria for testing. Our procedure renders it unlikely that a negative serum would be called positive but admits the possibility of a few weakly positive sera being called negative. Be that as it may, the specificity of the serum-virus reaction is further evidence that this virus is the specific agent responsible for the human disease, and finally, that the virus is different and the encephalitis in human beings is serologically distinct from others previously described.

Knowledge that antibodies persist for  $2\frac{1}{2}$  years in the blood of convalescents is an aid in searching for an endemic focus and in mapping out the time and space spread of the virus. Thus far, we know that the disease appeared in Paris, Illinois, in 1932, and was present in 1933 and 1934 in the north central states and New York.

#### CONCLUSIONS

1. A protection test for measuring serological protective properties against the encephalitis (St. Louis type) virus is described.
2. Normal non-contact sera and sera from persons supposed to have had no exposure to the disease do not protect against the virus. 82.5 per cent of sera from tested St. Louis encephalitis convalescents and at least 66 per cent of sera from tested persons thought to have had the disease do show protective properties.
3. The protective activity of sera is maintained for at least  $2\frac{1}{2}$  years after onset of the disease. *In vitro* aging of serum decreases its activity.
4. Protection tests indicate that the virus was present as early as 1932 in Paris, Illinois, spread through the north central states and reached New York in 1933, and was again active in the north central states in 1934.

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# THE EMIGRATION OF PNEUMOCOCCI TYPE III FROM THE BLOOD INTO THE THORACIC DUCT LYMPH OF RABBITS, AND THE SURVIVAL OF THESE ORGANISMS IN THE LYMPH FOLLOWING INTRAVENOUS INJECTION OF SPECIFIC ANTISERUM

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Various workers have shown that large colloidal particles such as hemoglobin, and other soluble proteins, as well as certain dyes, pass readily from the blood stream to the lymphatics. It has also been repeatedly demonstrated that various insoluble materials including bacteria are absorbed into lymphatics from local sites, such as the cutaneous and subcutaneous tissues, peritoneum, pleura, etc. To the best of our knowledge little or no data exist concerning the penetration into the lymph of bacteria introduced into the blood stream. The first part of this paper reports the results of experiments designed to obtain information on this point. Having determined the fact that pneumococci do emigrate from the blood stream to the lymphatics, we were led to investigate the effect of specific antisera, intravenously injected, on the bacteria present in the lymph. This seemed pertinent inasmuch as there is a lack of information dealing with the effect of antibody on organisms after their arrival in the lymphatics.

A number of studies are, however, available upon the relative amounts of antibodies in blood and in lymph in non-infected animals. These have been reviewed in sufficient detail by one of us (1). They indicate that the antibody content of lymph is invariably lower than that of blood, and that the antibody concentration is relatively proportional to the protein content of the fluids examined. The fact that McMaster and Hudack (2) have recently shown that for some days

after intradermal injection of an antigen, agglutinins induced by it can be found in higher concentration in extracts of regional lymph nodes than in the blood, does not negative the generalization that lymph is poorer in antibodies than blood since such extracts are derived from gland cells, lymph, and traces of blood. In a few of the infected animals studied by us we have made examinations for the presence of antibodies and their serum vehicle.

### *Materials and Methods*

The microorganism employed has been a Type III pneumococcus, Strain SV, which was used by Tillett (3) in his experiments on the infectivity of this organism for rabbits. The virulence for rabbits is such that an intradermal injection of 0.001 cc. of a 16 hour blood broth culture will kill the animal. Tillett showed that the organism produces a bacteremia leading to death in 1 to 5 days depending upon the dosage.

16 and 20 hour rabbit blood infusion broth cultures were used in the experiments which follow, rabbits being inoculated with large amounts intravenously for immediate observation of the transfer of organisms from blood to lymph, and with small amounts for the study of conditions on the following day. Measured samples of blood from the right jugular vein and of thoracic duct lymph were removed at intervals. Serial dilutions of these were plated by pouring horse blood agar plates.

In a number of experiments the effects of antisera in sterilizing the blood and in failing to sterilize the lymph were observed. Antisera of two sorts were used, *Pneumococcus* Type III rabbit antiserum prepared in the laboratory of Bacteriology, and *Pneumococcus* Type III horse antiserum prepared by the Massachusetts Antitoxin and Vaccine Laboratory.

The experimental rabbits were anesthetized by intravenous injections of nembutal in physiological saline, a fraction of the initial dose being repeated in order to maintain complete anesthesia throughout the experiment. In order to expose the thoracic duct, the left external jugular vein is followed to its junction with the subclavian vein. This is just above the clavicle which need not be removed, though a somewhat better exposure is secured if it is taken out. After tying all entering branches, the subclavian and jugular veins are ligated 2 cm. from the point of junction. Last of all, the subclavian vein is tied just central to the entrance of the thoracic duct which enters on the inner side of the junction of the veins. The duct is extremely delicate, and close dissection of it is apt to be disastrous. The venous pocket which has been formed is now opened and a cannula tied in it. When this is first done the lymph is usually bloody, the source of the blood being small veins which are often impossible to locate. In this latter case, a cannula is passed into the venous pocket and through it into the opening of the thoracic duct so as to be out of the region of entering veins. It is absolutely essential that the thoracic duct lymph have no direct contamination by blood.

Such lymph in the rabbit, as in other animals, almost invariably contains red cells which are normal constituents of liver and intestinal lymph arising from the highly permeable capillaries in these regions and not from direct communication between the thoracic duct and small veins.

*Emigration of Pneumococci from Blood Stream to the Lymphatics*

The results obtained in rabbits following the intravenous injection of pneumococci and subsequent cultivation of blood and lymph may be classified under three headings on the basis of the number of bacteria introduced and the time elapsing after injection.

(a) *Large Dose of Pneumococci; Immediate Examination; Organisms Enter the Lymph within a Brief Period.*—The following protocol and Fig. 1 illustrate the sequence of events following the intravenous inoculation of the organisms derived from 20 cc. of blood broth culture. They persist in the blood stream in large numbers and enter the lymph within an hour.

*Mar. 6, 1935.*—Rabbit, weight 2.6 kilos. Nembutal anesthesia. 12:50 p.m., Preparation finished. The thoracic duct lymph appears free from blood. Rectal temperature 101.8°F. 1:15, White cells in lymph 27,500 per c.mm.; red cells in lymph 700 per c.mm.

1:20, 20 cc. of 16 hour blood broth culture of SV concentrated to 0.8 cc. and injected in ear vein.

1:22, Blood culture 1, 57,000,000 pneumococci per cc. 1:26, Lymph culture 1, 0 pneumococci per cc. 1:28, White cells in lymph 26,900 per c.mm.; red cells in lymph 600 per c.mm. 1:45, Rectal temperature 102.4°F.

1:52, Blood culture 2, 13,000,000 pneumococci per cc. 1:55, Lymph culture 2, contaminated, 10 (?) pneumococci per cc. 1:57, White cells in lymph 32,200 per c.mm.; red cells in lymph 400 per c.mm. 2:00, Rectal temperature 102.3°F.

2:23, Blood culture 3, 7,000,000 pneumococci per cc. 2:24, Lymph culture 3, 40 pneumococci per cc. White cells in lymph 39,300 per c.mm.; red cells in lymph 300 per c.mm. 2:29, Rectal temperature 102.4°F.

3:40, Blood culture 4, 32,000,000 pneumococci per cc. Lymph culture 4, 2,700 pneumococci per cc. White cells in lymph 38,300 per c.mm.; red cells in lymph 300 per c.mm. 3:46, Rectal temperature 102.6°F.

4:23, Blood culture 5, 90,000,000 pneumococci per cc. 4:27, Lymph culture 5, 7,200 pneumococci per cc. White cells in lymph 36,000 per c.mm.; red cells in lymph 200 per c.mm. 4:34, Rectal temperature 102.8°F.

4:58, Blood culture 6, 280,000,000 pneumococci per cc. 5:02, Lymph culture 6, 24,000 pneumococci per cc. White cells in lymph 29,200 per c.mm.; red cells in lymph too few for count. 5:21, Rectal temperature 103.1°F. 5:33, Rectal temperature 103.0°F.



5:35, Blood culture 7, 230,000,000 pneumococci per cc. Lymph culture 7, 29,000 pneumococci per cc. White cells in lymph 31,200 per c.mm.; red cells in lymph 200 per c.mm. Experiment terminated.

The number of pneumococci per cubic centimeter of blood and lymph is shown in Fig. 1. The organisms in the lymph increased steadily but never became so numerous as in the blood.

(b) *Smaller Dose of Pneumococci; Immediate Examination; Organisms Markedly Reduced in Blood, Do Not Enter Lymph within 4½ Hours.*—When 1/20 of the dose of pneumococci used in the previous experiment was introduced, the bacterial count in the blood fell within 2 hours to 1 per cent of its original value. No organisms appeared in the lymph during the time of observation.

Feb. 1, 1935. Rabbit, weight 2.3 kilos. Nembutal anesthesia. 11:25 a.m., Thoracic duct isolated. Rectal temperature 99°F.

12:17 p.m., Injected 1.0 cc. 16 hour blood broth culture SV intravenously.

12:19, Blood culture 1, 153,000 pneumococci per cc. 12:23, Lymph culture 1, sterile (0.25 cc.).

12:47, Blood culture 2, 28,500 pneumococci per cc. 12:49, Lymph culture 2, sterile (0.25 cc.). 12:52, Rectal temperature 99°F.

1:07, Blood culture 3, 15,800 pneumococci per cc. 1:10, Lymph culture 3, sterile (0.25 cc.).

1:30, Blood culture 4, 5,000 pneumococci per cc. 1:34, Lymph culture 4, sterile (0.25 cc.). Rectal temperature 99°F.

2:02, Blood culture 5, 1,670 pneumococci per cc. 2:07, Lymph culture 5, sterile (0.25 cc.).

3:10, Blood culture 6, 1,410 pneumococci per cc. 3:14, Lymph culture 6, sterile (0.25 cc.). Rectal temperature 98.6°F.

3:45, Blood culture 7, 2,250 pneumococci per cc. 3:48, Lymph culture 7, sterile (0.25 cc.).

4:20, Blood culture 8, 1,570 pneumococci per cc. 4:24, Lymph culture 8, sterile (0.25 cc.). Rectal temperature 99.8°F.

4:51, Blood culture 9, 7,300 pneumococci per cc. 4:55, Lymph culture 9, sterile (0.25 cc.). 5:15, Rectal temperature 100°F. Experiment terminated.

(c) *Small Dose of Pneumococci; Examination after 20 Hours; Organisms Increasing in the Blood Stream and Lymph.*—Intravenous injection of 0.5 cc. of a 16 hour blood broth culture is followed after 20 hours by a bacteremia and the invariable presence of organisms in the lymph. This condition is apparent from the experiment recorded below, summarized in the form of a graph in Fig. 2, and from the

experiments which follow in which the effect of antisera on the pneumococci in the lymph was studied.

Mar. 26, 1935. Rabbit, weight 2.2 kilos. 5:00 p.m., Injected with 0.5 cc. of 16 hour blood broth culture of Strain SV. Rectal temperature 102.6°F.

Mar. 27. 9:00 a.m., Rectal temperature 104°F. Nembutal anesthesia. 9:50, Rectal temperature 100.8°F. 12:00 m., Thoracic duct cannulated. Lymph clear.

1:00 p.m., Blood culture 1, 23,000,000 pneumococci per cc. 1:05, Lymph culture 1, 3,300 pneumococci per cc. 1:06, Rectal temperature 99.2°F.

2:20, Lymph culture 2, 6,800 pneumococci per cc. 2:24, Blood culture 2, 43,000,000 pneumococci per cc.

3:20, Blood culture 3, 195,000,000 pneumococci per cc. 3:24, Lymph culture 3, 25,000 pneumococci per cc. 3:26, Rectal temperature 100.8°F. 3:27, Experiment terminated.

Throughout the experiment counts of the numbers of red blood corpuscles in the lymph were made on every sample; none were found.

The three preceding experiments indicate that *Pneumococcus* Type III virulent for rabbits passes from the blood stream into the lymphatic system where it increases. The rapidity with which this migration is accomplished appears to be related to the numbers of pneumococci injected, since only in the case of the intravenous administration of a large quantity of culture are the organisms found in the lymph within 4 hours. With smaller doses lymphatic infection is not observed within this interval. This fact, however, does not necessarily imply that organisms do not reach the lymph during the period immediately subsequent to their introduction into the blood; for if the number migrating into the lymph bears a more or less constant relation to the number in the circulating blood, then with smaller doses those entering the lymph would be too few to be detected when samples of 0.25 cc. are plated. However this may be, it is certain that at some time after the inoculation of small doses, pneumococci do enter the lymph, for they are found there regularly at 22 hours, where they multiply and may even become more numerous than in the blood.

*Effect of Specific Antiserum Injected Intravenously on Pneumococci Present in the Lymph*

Since it was established in the foregoing experiments that 20 hours after injection organisms were regularly demonstrable in the thoracic



duct lymph, it became of interest in view of the well known sterilizing effect of antiserum on pneumococci in the blood to observe the action of this agent on the organisms present in the lymph. With this in view we have studied the result of intravenous treatment of animals thus prepared, employing Antipneumococcus Type III rabbit and horse sera.

*(a) Effect of Antipneumococcus Type III Rabbit Serum on the Organisms in the Blood and Lymph 23 Hours after Infection.—*

Apr. 29, 1935. Rabbit, 2.3 kilos. 3:30 p.m., 0.05 cc. of 1:2 dilution of a 20 hour blood broth culture intravenously. Rectal temperature 103.5°F.

Apr. 30. 9:00 a.m., Rectal temperature 106.0°F. Nembutal anesthesia. 9:30, Rectal temperature 104.8°F. 10:40, Rectal temperature 103°F. 2:20 p.m., Preparation finished. White cells in lymph 23,000 per c.mm.; red cells in lymph too few to count.

2:25, Blood culture 1, 900,000 pneumococci per cc. Lymph culture 1, 1,300 pneumococci per cc. 2:30, Rectal temperature 103.2°F.

2:37, Lymph culture 2, 2,000 pneumococci per cc. 3:30, Blood culture 2, 1,500,000 pneumococci per cc. Lymph culture 3, 12,000 pneumococci per cc. 3:32, White cells in lymph 27,000 per c.mm.; red cells in lymph too few to count. Stained film of blood shows encapsulated organisms. 3:34, Rectal temperature 102.6°F.

4:29, Blood culture 3, 9,000,000 pneumococci per cc. 4:31, Lymph culture 4, 60,000 pneumococci per cc. White cells in lymph 12,400 per c.mm.; red cells in lymph 200 per c.mm.

4:53, 8.0 cc. Antipneumococcus Type III rabbit serum injected into an ear vein.

5:00, Blood culture 4, 15,000 pneumococci per cc. 5:02, Lymph culture 5, 27,000 pneumococci per cc. White cells in lymph 18,700 per c.mm.; red cells in lymph 300 per c.mm. 5:20, Rectal temperature 102°F.

5:50, Blood culture 5, 2,000 pneumococci per cc. 5:51, Rectal temperature 102.8°F. 5:54, Lymph culture 6, 15,000 pneumococci per cc. White cells in lymph 14,900 per c.mm. 5:59, Experiment terminated.

The number of pneumococci in blood and lymph is shown in Fig. 3. Here the sterilizing effect of the antiserum upon the blood and the comparative absence of effect upon the lymph are strikingly shown. The organisms in the blood are reduced approximately 4,500 times after administration of antiserum. In contrast only a fourfold reduction occurs in the number of pneumococci in the lymph. It is also interesting to observe that at this stage of the infection the numbers of bacteria both in the blood and in the lymph were rapidly increasing.

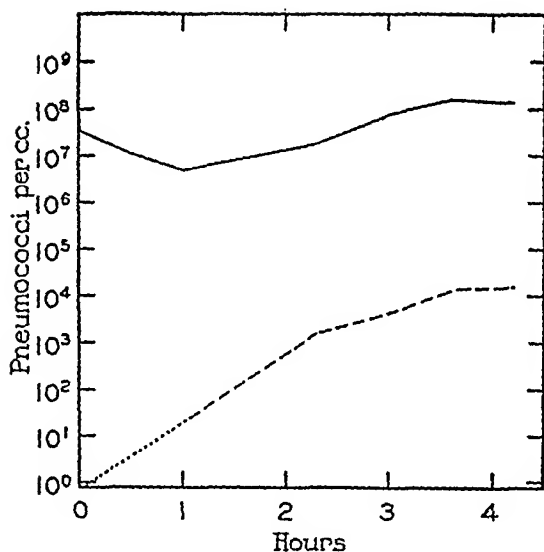


FIG. 1

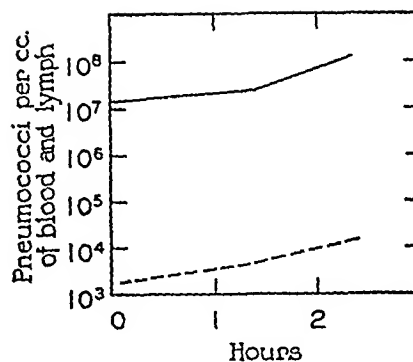


FIG. 2

FIG. 1. —, number of organisms in blood; - - -, number of organisms in lymph; ....., probable appearance of organisms in lymph.

FIG. 2. —, number of organisms in blood; - - -, number of organisms in lymph.

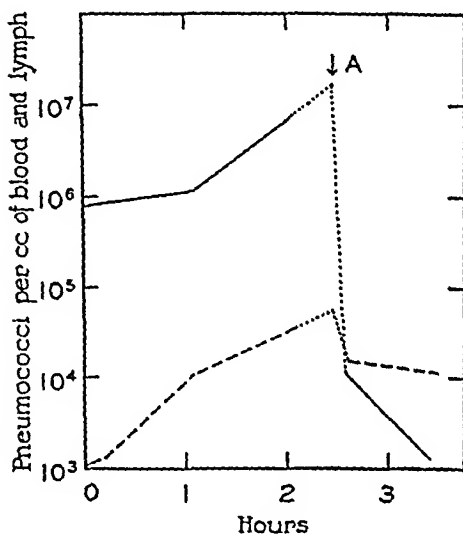


FIG. 3

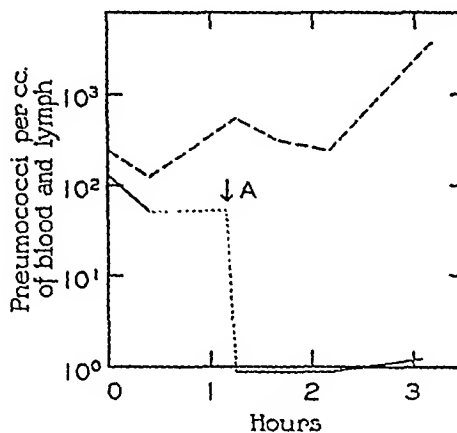


FIG. 4

FIG. 3. —, number of organisms in blood; - - -, number of organisms in lymph; ....., in blood and lymph curves, probable numbers of pneumococci before and at the time of antiserum injection. At A, 8.0 cc. of Antipneumococcus Type III rabbit serum were given intravenously.

FIG. 4. —, number of organisms of blood; - - -, number of organisms in lymph. At A, 10.0 cc. of Antipneumococcus Type III horse serum were given intravenously.

duct lymph, it became of interest in view of the well known sterilizing effect of antiserum on pneumococci in the blood to observe the action of this agent on the organisms present in the lymph. With this in view we have studied the result of intravenous treatment of animals thus prepared, employing Antipneumococcus Type III rabbit and horse sera.

(a) *Effect of Antipneumococcus Type III Rabbit Serum on the Organisms in the Blood and Lymph 23 Hours after Infection.*—

Apr. 29, 1935. Rabbit, 2.3 kilos. 3:30 p.m., 0.05 cc. of 1:2 dilution of a 20 hour blood broth culture intravenously. Rectal temperature 103.5°F.

Apr. 30. 9:00 a.m., Rectal temperature 106.0°F. Nembutal anesthesia. 9:30, Rectal temperature 104.8°F. 10:40, Rectal temperature 103°F. 2:20 p.m., Preparation finished. White cells in lymph 23,000 per c.mm.; red cells in lymph too few to count.

2:25, Blood culture 1, 900,000 pneumococci per cc. Lymph culture 1, 1,300 pneumococci per cc. 2:30, Rectal temperature 103.2°F.

2:37, Lymph culture 2, 2,000 pneumococci per cc. 3:30, Blood culture 2, 1,500,000 pneumococci per cc. Lymph culture 3, 12,000 pneumococci per cc. 3:32, White cells in lymph 27,000 per c.mm.; red cells in lymph too few to count. Stained film of blood shows encapsulated organisms. 3:34, Rectal temperature 102.6°F.

4:29, Blood culture 3, 9,000,000 pneumococci per cc. 4:31, Lymph culture 4, 60,000 pneumococci per cc. White cells in lymph 12,400 per c.mm.; red cells in lymph 200 per c.mm.

4:53, 8.0 cc. Antipneumococcus Type III rabbit serum injected into an ear vein.

5:00, Blood culture 4, 15,000 pneumococci per cc. 5:02, Lymph culture 5, 27,000 pneumococci per cc. White cells in lymph 18,700 per c.mm.; red cells in lymph 300 per c.mm. 5:20, Rectal temperature 102°F.

5:50, Blood culture 5, 2,000 pneumococci per cc. 5:51, Rectal temperature 102.8°F. 5:54, Lymph culture 6, 15,000 pneumococci per cc. White cells in lymph 14,900 per c.mm. 5:59, Experiment terminated.

The number of pneumococci in blood and lymph is shown in Fig. 3. Here the sterilizing effect of the antiserum upon the blood and the comparative absence of effect upon the lymph are strikingly shown. The organisms in the blood are reduced approximately 4,500 times after administration of antiserum. In contrast only a fourfold reduction occurs in the number of pneumococci in the lymph. It is also interesting to observe that at this stage of the infection the numbers of bacteria both in the blood and in the lymph were rapidly increasing.

*(b) Effect of Antipneumococcus Type III Horse Serum on the Organisms in the Blood and Lymph 22 Hours after Infection.—*

May 15, 1935. Rabbit, weight 2 kilos. 4:30 *p.m.*, Inoculation intravenously with 0.1 cc. of 1:2 dilution of 20 hour blood broth culture of SV. Temperature at time of inoculation 103.4°F.

May 16. 9:00 *a.m.*, Rectal temperature 102.4°F. 2:20 *p.m.*, Preparation finished. Blood culture 1, 185 pneumococci per cc. Lymph cultures 1 and 2, average 450 pneumococci per cc. White cells in lymph 35,000 per c.mm.; red cells in lymph 500 per c.mm. 2:30, Rectal temperature 100.6°F.

2:45, Blood culture 2, 75 pneumococci per cc. Lymph culture 3, 180 pneumococci per cc. White cells in lymph 40,000 per c.mm.; red cells in lymph 500 per c.mm.

3:30, 10 cc. Antipneumococcus Type III horse serum injected intravenously.

3:35, Blood culture 3, 0 pneumococci per cc. Lymph culture 4, 770 pneumococci per cc. 3:37, Specimen of lymph taken for antibody titration.

3:40, Blood culture 4, 0 pneumococci per cc. White cells in lymph 67,000 per c.mm.; red cells in lymph 500 per c.mm.

3:58, Blood culture 5, 1 (?) pneumococcus per cc. Lymph culture 5, 560 pneumococci per cc. White cells in lymph 43,000 per c.mm. Red cells in lymph 400 per c.mm.

4:30, Blood culture 6, 0 pneumococci per cc. Lymph culture 6, 450 pneumococci per cc. White cells in lymph 36,600 per c.mm.; red cells in lymph 300 per c.mm. 5:05, Lymph sample 7 (not cultured).

5:30, Blood culture 7, 2 pneumococci per cc. Lymph culture 8, 6,000 pneumococci per cc. White cells in lymph 38,000 per c.mm.; red cells in lymph 1,500 per c.mm. Experiment terminated.

The course of the experiment is shown in Fig. 4. It is of interest that at the beginning of observation, approximately 22 hours after blood inoculation, there were more pneumococci in the lymph than in the blood. The injection of antiserum sterilized the blood for a period of almost 2 hours but had no effect on the number of organisms in the lymph, which remained practically stationary and then increased.

*Relative Quantities of Antibody in Blood and Lymph in Infected Rabbits*

In the experiments involving the use of antiserum, titrations of the agglutinin content of lymph and in some cases that of the blood serum were carried out. Within 10 minutes after injection of 8 cc. of Antipneumococcus Type III rabbit serum (see page 855), the agglutinin

titre of which was approximately 1:200, that of the rabbit's blood serum rose to 1:32 and did not increase further. No agglutination occurred in any of the lymph samples in 1:2 dilution. It is of interest that the presence of soluble antigenic substance in the blood serum

TABLE I

*Serological Reactions of Blood Serum and Lymph of a Rabbit Injected Intravenously with Antipneumococcus Type III Horse Serum\**

| Samplings of blood and lymph | Time of sampling | Agglutinin titre rs. Pneu-mococcus III† | Precipitinogen titre (horse serum) rs. anti-horse rabbit serum‡ | Precipitinogen titre (specific soluble pneu-mococcus antigen) | Remarks   |
|------------------------------|------------------|---|---|---|---|
|                              | <i>hrs. min.</i> |   |   |   |   |
| Blood 1                      | 0 00             | 0                                       | 0   | 1:8   | 15 cc. Antipneumococcus III horse serum given intravenously 2 min. before taking blood sample 3 |
| " 2                          | 0 25             | Not done                                | Not done  | Not done  |   |
| " 3                          | 0 50             | 1:128                                   | 1:128   | " "   |   |
| " 4                          | 1 12             | 1:128                                   | 1:128   | " "   |   |
| " 5                          | 2 27             | 1:128                                   | Not done  | " "   |   |
| " 6                          | 2 55             | 1:128                                   | " "   | " "   |   |
| " 7                          | 3 25             | 1:128                                   | " "   | " "   |   |
| " 8                          | 3 56             | 1:128                                   | " "   | " "   |   |
| " 9                          | 4 22             | 1:128                                   | 1:32  | 1:8   |   |
| Lymph 1                      | 0 10             | 0                                       | 0   | 1:8   | Lymph turbid; ring test difficult to read, in case of specific soluble pneumococcus antigen     |
| " 2                          | 0 30             | 0                                       | 0   | Not done  |   |
| " 3                          | 0 55             | 0                                       | 0   | " "   |   |
| " 4                          | 1 15             | 1:8                                     | 1:32  | " "   |   |
| " 5                          | 2 30             | Not done                                | 1:32  | " "   |   |
| " 6                          | 3 00             | 1:32                                    | 1:32  | " "   |   |
| " 7                          | 3 30             | Not done                                | 1:32  | " "   |   |
| " 8                          | 4 00             | 1:32                                    | 1:32  | " "   |   |
| " 9                          | 4 30             | Not done                                | 1:32  | 1:8   |   |

\* 15 cc. of the antiserum were injected.

† Agglutinin titre of Antipneumococcus Type III horse serum injected was 1:512.

‡ Precipitin titre of anti-horse rabbit serum against falling dilutions of horse serum was 1:1,000.

could be demonstrated to a titre of 1:8 using Antipneumococcus Type III horse serum as antibody.

In the experiment recorded above in which 10 cc. of Antipneumococcus Type III horse serum exhibiting an agglutinin titre of 1:128 was



## PNEUMOCOCCI TYPE III IN BLOOD AND LYMPH

injected, no agglutinins were detected in any of the lymph samples, but the presence of the horse serum in low concentration in the lymph was revealed by titration with anti-horse rabbit serum. The agglutinin content of the blood serum was not determined. 15 cc. of an Anti-pneumococcus Type III horse serum having an agglutinin titre of 1:512 were injected in a second experiment in all other respects similar to the foregoing. Agglutinins were found in the lymph to a titre of 1:32 and in the blood to 1:128. Nevertheless, while the organisms occurring in large numbers in the blood previous to administration of serum were almost completely eliminated, the numbers in the lymph, which were nearly equivalent to those in the blood, were at first slightly reduced and then began to increase. Again free soluble pneumococcus antigen was shown to coexist in the blood serum along with a considerable amount of agglutinin. A summary of the serological observations in the case of this rabbit is presented in Table I.

From these results taken as a whole it is clear that when blood and lymph infection with *Pneumococcus* Type III occurs, a very large quantity of an antiserum containing a high titre of agglutinins is required before these antibodies become demonstrable in the lymph. This volume of serum has been estimated to represent about one-seventh of the blood volume of the rabbit. If the quantity of either heterologous or homologous antiserum is less and the agglutinin titre lower, antibody fails to appear in the lymph. Since even when antibody does enter the lymph, the organisms are not eliminated or significantly reduced in number, it is suggested that a second factor, possibly one involving phagocytic cells is absent or inoperative.

## DISCUSSION

The import of these experiments is apparent, and from the point of view of therapy, disturbing. It is first of all clear that somehow or other microorganisms in the blood pass through the capillary walls into the tissue fluid and through the walls of the lymph capillaries to enter the lymph stream. This passage requires neither motility on the part of the organisms nor phagocytosis, and it is accomplished rapidly. Our experiments have dealt with thoracic duct lymph alone and the major part of this lymph, indeed in the quiescent anesthetized animal practically all of it, has origin in the highly permeable capil-

laries of the abdominal region, rather than in the capillaries of the skin, subcutaneous tissues, and muscles. No data are available for the lymph from these regions. If bacteria were present in such lymph, their growth would undoubtedly be less inhibited by antibody action than in lymph from the abdominal region, owing to the fact that the less permeable blood capillaries of the periphery permit but slight passage of antibodies (1). In the presence of a bacteremia, apparently one cannot expect to sterilize the lymph by a single intravenous injection of antiserum. If the injection sterilizes the blood, organisms will begin at once to enter from the lymph and removal from the blood will depend upon a sustained concentration of antibodies due to a very large initial injection or to subsequent small injections.

The problem of effecting the removal of the pneumococci in the lymph itself might possibly be attacked by some measure leading to an increase in the permeability of the blood capillaries, thus raising the antibody content of the lymph. The organization, however, of the lymphatic system both in the multiplicity of vessels and in the complexity of the nodes makes it difficult to think that lymph antibody content and lymph flow can be increased sufficiently to bring about sterilization, but it is of course worth while to make the attempt experimentally. Moreover, if antibody were increased in some manner, it is exceedingly doubtful whether its presence alone would be sufficient for the efficient elimination of the organisms in the lymph, for in one instance mentioned above a considerable quantity failed to produce any but a transitory diminution in the number of pneumococci. We believe, therefore, that the adjuvant action of one or more additional factors, most probably cellular in nature, is requisite.

It must be emphasized that the foregoing remarks are based upon observations made during only the 4 hours subsequent to the administration of antiserum. It is entirely possible that after this time mobilization of auxiliary defensive mechanisms may take place, resulting in the destruction of the organisms in the lymphatic area.

#### SUMMARY

1. Rabbits injected intravenously with a large dose of a virulent Type III Pneumococcus develop a bacteremia, and within an hour organisms may be cultivated from the thoracic duct lymph. The

rapidity with which entrance into the lymph occurs appears to be correlated with the size of the dose injected.

2. The organisms may become more numerous in the lymph than in the blood.

3. If homologous or heterologous antisera are injected, the blood may be sterilized, but though the organisms may be lessened in the lymph, sterilization at least within 4 hours is not secured, and in the intact animal living organisms must continue to enter the blood with the thoracic duct lymph.

4. In infected rabbits after intravenous injection of considerable quantities of antisera containing moderate amounts of agglutinin, no antibody appears in the thoracic duct lymph although the presence of horse serum may be detected.

The injection of a very large quantity of antiserum containing a high titre of agglutinin is followed by the penetration of antibody into the lymph. This, however, has failed to sterilize the lymph or to permanently affect the rate of multiplication of the pneumococci.

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